Prevalence and Distribution of Antibiotic Resistant Bacteria in Surface Waters That Are Sources of Drinking Water in Rural South Island, New Zealand

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by

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Abstract

In this thesis, I describe a hypothetical journey of a tourist in New Zealand who has arrived on an airplane and is thirsty to explore the country's spectacular rural and remote environments. My tourist sometimes will have to find her own drinking water. Surface waters in rural areas may be contaminated by waste from livestock, septic tanks, and wastewater pipes. These contaminants can include antibiotics and antibiotic resistant bacteria. When these surface waters are used for drinking and food preparation, they may expose users to antibiotic resistant pathogens, reducing treatment options and delivering poorer treatment outcomes. I was interested in whether these surface waters that are used for drinking water in various rural South Island locations harboured multidrug resistant (MDR) bacteria, and whether associations between resistance and location could be observed.

The microbiological quality of water on long haul international and domestic flights was compared using *E. coli* as an indicator organism. Next, the number and diversity of MDR *E. coli* was measured and compared in rural areas across three water sources; treated water (Treated), untreated stream water (Stream), other untreated surface waters such as rivers and tributaries (Other). Comparisons were made between the Marlborough, Banks Peninsula and Wider Canterbury areas. Through a case study in Okains Bay I observed whether there were seasonal effects on MDR *E. coli* in the main drinking water sourced from the Opara Stream.

E. coli was below the detection limit in the airplane water analysis, however, water from long haul international flights had significantly poorer microbiological quality than domestic airplanes. Across the three regions, 15% of tested isolates were MDR. Among the MDR isolates, 1% were isolated from Treated, 46% were isolated from Other, and 53% were isolated from Stream. No antibiotic enrichment was needed to identify MDR *E. coli* from water samples. The *E. coli* population density was not predictive of resistant E. coli occurrence in the environment. In the Okains Bay case study, MDR *E. coli* were detected at similar frequencies between the sites along the Opara Stream across the four seasons. The residential home had significantly higher MDR frequencies than all the Opara Stream sites.

36% of isolates that were resistant to ampicillin or ciprofloxacin were phenotypically confirmed to be ESBL or AmpC producers. 64% of tested isolates were able to share at least one resistance through horizontal gene transfer. Genetic analysis of 20 isolates indicated that a combination of point mutations, efflux systems and AmpC β–lactamases are the putative contributors to resistance phenotypes observed in the sequenced isolates.

Previous studies have determined that *E. coli* concentrations in rural surface waters are often above what is considered safe for drinking. Nevertheless, few studies have investigated the presence of MDR bacteria. The results from my analysis suggest that MDR *E. coli* and ESBL- and AmpC-producing *E. coli* are present in surface waters in Marlborough, Banks Peninsula, and the Wider Canterbury area. These MDR *E. coli* are able to share resistance genes with susceptible bacteria. An important finding with relevance to community safety is that the exposure to, and risk of, catching an infection from a MDR *E. coli* cannot be estimated using surveys of total *E. coli* concentrations.

Abbreviations

µg	Microgram
µL	Microliter
AMR	Antimicrobial resistance
Amp	Ampicillin
βL	β-lactamase
BLI	β-lactamase inhibitor
BP	Banks Peninsula
C	Canterbury
Caz	Ceftazidime
cfu	Colony forming unit
Chl	Chloramphenicol
Cip	Ciprofloxacin
Cla	Clavulanic acid
COVID-19	Coronavirus disease of 2019
Ctx	Cefotaxime
DoC	Department of Conservation
DWSNZ	Drinking Water Standards for New Zealand
ESBL	Extended-spectrum beta-lactamase
ESR	Institute of Environmental Science and Research
Gen	Gentamicin
GLM	Generalized linear model
GLMM	Generalized linear mixed model
Kan	Kanamycin
MDR	Multidrug resistance
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Marlborough Sounds
PDR	Pandrug resistant
R2A	Reasoner's 2A

Rif	Rifampicin
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
Tet	Tetracycline
TBX	Tryptone Bile-X-glucuronide
Tmp	Trimethoprim
VRE	Vancomycin-resistant *Enterococcus*
WHO	World Health Organisation
XDR	Extensively drug resistant

Chapter 1: Antibiotics and resistance

1.1 Antibiotics and resistance

Antibiotic resistant bacteria are now commonly found in New Zealand's surface waters (Schousboe et al., 2015; Van Hamelsveld et al., 2019; Winkworth-Lawrence & Lange, 2016), and the contribution of the environmental exposures to antibiotic resistant bacteria for human health is gaining attention (Karkman et al., 2019). Both here and internationally people are concerned (Ministry of Health & Ministry for Primary Industries, 2017a; World Health Organisation, 2014). This research was designed in response to those concerns. Primarily, it is an investigation into the occurrence and diversity of antimicrobial resistance (AMR) across the Marlborough, Banks Peninsula and Wider Canterbury areas at locations whose main drinking water was sourced from a nearby fresh surface water.

Increasing amounts of antibiotics/antimicrobial agents are being introduced into the environment. They are used, for example, in dairy farming where they are being linked with multidrug resistant (MDR) bacteria (Collis et al., 2019), through human therapeutics where the misuse and overuse of antibiotics have been associated with MDR pathogens (Davies & Davies, 2010), and even through veterinary medicine (Teuber, 2001). People may be exposed to resistant bacteria through different forms of contact with water. We drink it and play in it; we touch our companion animals after they swim in it; we harvest food from it. This thesis describes my findings. Firstly, I measured water quality by enumerating the bacterium *Escherichia coli*. Secondly, I determined the frequency of MDR *E. coli*. Thirdly, I determined the potential for *E. coli* to spread resistant determinants through horizontal gene transfer. Finally, I used whole genome sequencing of selected isolates to infer the underlying genetic basis of resistance.

Antibiotic resistance is a global crisis. In the United States, around 3 million people a year are infected with a high-priority antibiotic resistant pathogen. Resistant isolates requiring a public health investigation are detected every 4 hours (U.S. Centers for Disease Control and Prevention, 2019). On the global scale, over 700,000 deaths annually are attributed to antibiotic resistant infections (O'Neill, 2016). The World Health Organisation (WHO) estimates that this number will be over 10 million by 2050 (Tagliabue & Rappuoli, 2018).

Resistance to the watershed antibiotic penicillin was detected in bacteria that previously had been susceptible within two years of its discovery (Walsh, 2003). Since then, the successive deployment of new antibiotics has been followed by the emergence of resistant strains (Manohar et al., 2020), with clinically significant resistance to novel antibiotics observed within a few months to a few years (Neu, 1992). Antibiotic resistance is common in nature, but prior to the human adoption of antibiotics in medicine and agriculture, resistant bacteria species that cause disease in people and companion animals was rare or absent. The use of antibiotics has rapidly sped up this evolution, contributing to the antibiotic 'resistome' in the environment (McEwen & Collignon, 2018). The antibiotic 'resistome' describes the collection of bacterial antibiotic resistance genes and all other resistance genes (Wright, 2007). Bacteria can encounter antibiotics during their use as chemotherapy, usually for bacterial infections. Antibiotic treatment and dosage in humans are determined by pharmacokinetic and pharmacodynamic models (Kurenbach et al., 2015), and these models are based on information measured *in vitro* (Steinkraus et al., 2007). Bacteria also encounter antibiotic residues in the environment. The contamination of antibiotic residues into environments such as surface waters can create a selective environment which can lead to overexpression of bacterial defensive systems such as efflux pumps and can select for bacteria that have mutations which allow them to survive (Lupo et al., 2012).

Bacterial resistance can be intrinsic, adaptive or acquired (Arzanlou et al., 2017). Intrinsic resistance mechanisms can occur through inherent structures or functions that allow the bacterium to tolerate antibiotics, for example, the selective permeability of the membrane or efflux pumps (Mazzariol et al., 2000). In this way, Gram-negative bacteria are intrinsically more resistant to antibiotics than Gram-positive bacteria due to the differences in the membrane structures (Zgurskaya et al., 2015). Transient bacterial resistance can occur through exposures to sub-lethal concentrations of antibiotics, and as the concentrations increase, high-level resistance can be observed. This type of adaptive resistance is governed by epigenetic inheritance and inducible gene expression (Motta et al., 2015). Acquired resistance describes two kinds of origins: horizontal gene transfer and mutation. The movement of mobile genetic elements such as plasmids and integrons from resistant bacteria via horizontal gene transfer has been identified as one of the significant contributors to the dissemination of antibiotic resistant bacteria in the environment (Dantas & Sommer, 2012), with plasmid-mediated conjugation being amongst the

most important routes (Davies, 1994). For instance, the efflux gene *tetL* associated with tetracycline resistance is found on conjugative plasmids (Chopra & Roberts, 2001). Another manner of acquiring resistance is through mutations in the bacterial genome which reduces the antibiotic's affinity for the target enzyme, for example, resistance to the antibiotic rifampicin can occur from a mutation in the *rpoB* subunit of RNA polymerase (Jin & Gross, 1988).

In the past few decades, the emergence of MDR, extensively drug-resistant (XDR) and pandrug-resistant (PDR) bacteria have caused increased concern as they pose a significant threat to human therapeutics and limit the current antibiotic treatments that we use to combat infections (Magiorakos et al., 2012). MDR is described as resistance to at least one antibiotic in three or more antibiotic categories, XDR is described as resistance to all but two or fewer antibiotic categories, and PDR is described as resistance to all antibiotics in all antibiotic categories (Magiorakos et al., 2012). On a global scale, extended-spectrum β-lactamase (ESBL)-producing bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA), and vancomycin-resistant enterococci (VRE) have become of particular concern from a healthcare perspective as they are able to confer resistance to wide array of antibiotics and can lead to poor treatment outcomes (Hassoun et al., 2017; Murray, 1997; Paterson & Bonomo, 2005).

1.2 Use and surveillance of antibiotics in society

Antibiotic resistant bacteria have been a global issue that has existed in humans, animals, food and the environment for decades (Davies & Davies, 2010). Understanding the patterns and frequency of antibiotic use and antibiotic resistant organisms in society and in the environment allows monitoring agencies and governments to fulfil their roles in antimicrobial stewardship. The aim of antimicrobial stewardship is to protect people from untreatable infections but also to preserve the effectiveness of antibiotics in the future (Best Practice Advocacy Centre New Zealand and National Institute for Health and Care Excellence, 2017).

The use of antibiotics is common in agriculture and in human and veterinary medicine. Concerns surrounding antibiotic resistance encompasses all three areas, with the misuse or overuse of antibiotics in each sector spreading resistant bacteria and resistant determinants within and between each sector (McEwen & Collignon, 2018). Links between human, animal (veterinary)

and food-producing animal diseases have been identified since the 19th century, which encouraged the 'One Health' approach, where the similarities between human and animal antibiotic uses were identified, and it was realized that collaboration across policy makers, clinicians and researchers were needed to prevent and control zoonotic diseases (Cassidy, 2017).

The exact quantity of antibiotics used in food production is difficult to measure. However, in 2017, it was estimated that on a global scale 93,309 tonnes of antibiotics was used in food-producing livestock, and it was predicted that by 2030, this number would rise to 104,079 tonnes (Tiseo et al., 2020). Food-producing livestock and humans often use the same classes of antibiotics for therapy, which means that there is a high risk of the emerging resistant bacteria to cause infections in both humans and animals (World Health Organisation, 2014). In New Zealand, the sale of antibiotics in agriculture and veterinary medicine is monitored. Antibiotic use in food-producing animals is regulated under the Agricultural Compounds and Veterinary Medicines Act 1997, which permits the use of agricultural compounds (i.e., antibiotics) only if their benefit outweighs the risk from residues for humans and the environment (Ministry for Primary Industries, 2020). Antibiotics prescribed by veterinarians are used throughout New Zealand for therapeutic and non-therapeutic uses (Schallenberg & Armstrong, 2004). McDougall et al. (2017) found that New Zealand farmers had little knowledge around the occurrence of antibiotic resistant bacteria, let alone MDR bacteria. Most farmers in the study relied on their veterinarians' advice regarding dosage and treatment, with dosage over the *in vitro* minimum inhibitory concentration (MIC) only considered in 21% of the cases. The most common antibiotic used in dairy and meat production are penicillins (Bryan & Hea, 2017). Nevertheless, resistances to other classes of antibiotics such as aminoglycosides (gentamicin, neomycin and streptomycin), quinolones (ciprofloxacin) and tetracyclines have been documented (Nulsen et al., 2008).

Currently in New Zealand, there are guidelines in place for antibiotic use in medicine, however, they are specific to each healthcare organization. That creates inconsistencies at a national level (Ministry of Health & Ministry for Primary Industries, 2017b). Antibiotic use for medicine in New Zealand is high in comparison to other countries, with 85-95% of antibiotics dispensed within the community and the remaining dispensed through hospital prescriptions (Duffy et al.,

2018; Williamson, 2016). Between 2004 and 2014, the community-based consumption of antibiotics increased by 46% (Williamson et al., 2016). The threat of antibiotic resistant pathogens is recognized, however, and through a 'One Health' approach, the 'Antimicrobial Resistance Action Planning Group' with representatives from human health, veterinary health and agriculture was created to implement a 'National AMR Action Plan' (Ministry of Health & Ministry for Primary Industries, 2017b). And although antibiotic resistance frequencies are not monitored as regularly as water quality, the Ministry of Health does fund the Institute of Environmental Science and Research (ESR) to undertake national AMR surveillance using data from various surveillance systems and sources (Ministry of Health & Ministry for Primary Industries, 2017b).

On a global scale, antibiotic resistance is also monitored. In 2015, the WHO launched the Global Antimicrobial Resistance Surveillance System (GLASS), which was the first system that collected resistance data globally from selected human pathogens (World Health Organisation, 2020c). Their aim is to *"..ensure that countries can design cost effective, evidence-based AMR response strategies that are prioritized for impact, in the context of whole of society engagement across the One Health spectrum."* Networks that contribute data to GLASS include the Central Asian and European Surveillance of Antimicrobial Resistance (CAESAR) network, which has identified the widespread network of antibiotic resistance, as well as patterns of specific resistance in clinical settings (World Health Organisation Regional Office for Europe, 2019) and the Centers for Disease and Controls (CDC) who collect health data to monitor the burden of antimicrobial resistance on healthcare (Centers for Disease Control and Prevention, 2020). Other surveillance networks include the European Antimicrobial Resistance Surveillance Network (EARS-Net), which is the largest publicly funded antibiotic resistance surveillance system in Europe (European Centre for Disease Prevention and Control, 2020a). This network collects and collates temporal and spatial antibiotic resistance data across Europe to inform health authorities and to support them in their policy and decision-making surrounding antibiotic resistance. As a part of the One Health approach, antibiotic resistance in food-producing animals is also monitored. The European Medicines Agency (EMA) works in collaboration with the European Food Safety Authority (EFSA) and the ECDC, and it is also involved in the European

Surveillance of Veterinary Antimicrobial Consumption (European Centre for Disease Prevention and Control. et al., 2017).

The recent COVID-19 pandemic has created further concern surrounding the misuse and overuse of antibiotics. Antibiotics have been prescribed to patients who have been infected with SARS-CoV-2 to prevent or treat bacterial co-infection. A study conducted by the European branch of the WHO indicated increased use of antibiotics during the COVID-19 pandemic, with 79-96% of uninfected participants taking antibiotics as a preventative measure, and 75% of infected participants receiving antibiotics even though only 15% of infected participants developed a bacterial co-infection (European Centre for Disease Prevention and Control, 2020b). These findings are also supported by Rawson et al. (2020) who identified that 72% of patients hospitalised with SARS-CoV-2 infection received broad spectrum antibiotics when only 8% were reported to have a bacterial coinfection.

In countries such as the United States, Germany and Italy, increased SARS-CoV-2 and MDR bacterial co-infection has been reported (Kampmeier et al., 2020; Nori et al., 2020; Porretta et al., 2020; Tiri et al., 2020). However, increased co-infection frequencies were not found in France and Spain (Contou et al., 2020; Garcia-Vidal et al., 2021). The overarching concern is that antimicrobial stewardship may be undermined due to the continuously increasing workload that healthcare workers face during this pandemic, and due to the prioritisation of COVID-19-related issues (Clancy & Nguyen, 2020). A combination of factors makes it difficult to formulate guidelines around the prescription of antibiotics in patients infected with SARS-CoV-2 for either prevention or treatment of bacterial coinfection. For one, the reporting methodology of microbiological data is inconsistent between countries, and the definitions for coinfection are variable, with lack of distinction between infections acquired from the community or from a hospital setting (World Health Organisation, 2020b).

The drivers behind antibiotic use are complex and differ by country. The differences in consumption of antibiotics can be society-, climate-, and socio-economic group-specific (Blommaert et al., 2014; Heinemann & Goven, 2006; Klein et al., 2018; Marra et al., 2010; Touboul-Lundgren et al., 2015). For example, where antibiotics can be bought without

prescription the antibiotic resistance crisis is much worse (World Health Organisation, 2020a). As mentioned earlier, in New Zealand, there are guidelines in place for antibiotic use, however, they are specific to each healthcare organisation (Ministry of Health & Ministry for Primary Industries, 2017a). This type of organisation may make it difficult to combat antibiotic resistance as a nation.

1.3 Sources of AMR in New Zealand

On a global scale, AMR in New Zealand is low, however, a rising trend in antibiotic resistant infections has been observed (Ministry of Health & Ministry for Primary Industries, 2017a). This rising trend has been attributed to various causes. The inappropriate use of antibiotics is a contributor (Williamson & Heffernan, 2014), as well as transmission of resistant organisms within hospital and community settings (Drinkovic et al., 2015; Graves et al., 2003). Anthropogenic modification of land has also aided in the distribution of resistant determinants in the environment (Asante & Osei Sekyere, 2019). In New Zealand, antimicrobial stewardship includes rational use of antibiotics to minimise selective pressure, and to practice effective infection control measures (Ministry of Health, 2007a).

According to surveys of sewage systems, New Zealand has one of the lowest levels of antibiotic resistance in the world (Hendriksen et al., 2019), however, investigations into other potential vectors of antibiotic resistant bacteria have indicated otherwise. Cooke (1976) identified MDR coliform bacteria in potable water supplies and discovered that transfer of resistance genes occurred within 2 hours of interaction between the MDR bacterium and a susceptible strain. Other water sources such as the Waimakariri river (Schousboe et al., 2015), Silverstream and the Ōtākaro/Avon river (Adewale, 2018; Van Hamelsveld et al., 2019) have also been found to harbour MDR coliforms. Currently, there are no standardised monitoring systems in place that specifically observe for antibiotic resistant bacteria within the New Zealand environment.

The use of antibiotics in agriculture and in medicine and veterinary treatment can lead to antibiotic resistance in the environment through the excretion of urine and faeces (Figure 1). The introduction of antibiotic residues to the environment through waste matter can contribute to antibiotic resistant pathogens by creating a fitness advantage for those less susceptible to

excreted antibiotics (Lundborg & Tamhankar, 2017; Van Epps & Blaney, 2016). Correlations between antibiotic use and the frequency and patterns of resistance in areas impacted by anthropogenic activity have been identified (Asante & Osei Sekyere, 2019). The antibiotic residues from such activities can be introduced into surface or subterranean waterways through urine and waste from livestock or septic tanks or wastewater pipes (Figure 1).

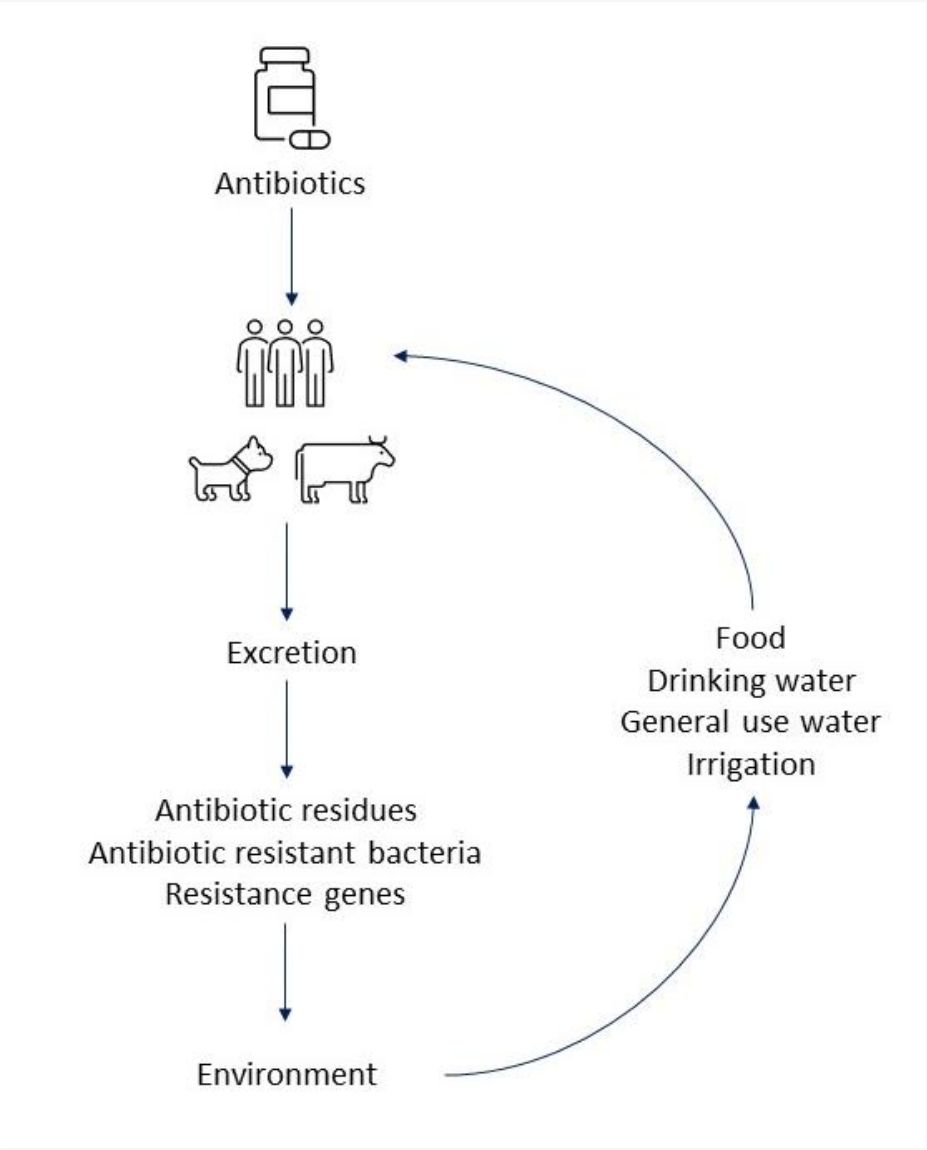


Figure 1 The cycle of antibiotic use and the pathway back to humans and animals.

1.3.1 Hospital and community

Transmission of MDR pathogens between people can occur through direct or airborne contact (Royal Society Te Apārangi, 2017). This was observed in New Zealand hospitals in the 1950s and 1960s, where healthcare workers were unknowingly spreading antibiotic resistant *Staphylococcus aureus* between their patients (Jowitt, 2019). In New Zealand, the three main pathogens of concern are community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA), Enterobacteriaceae and multidrug-resistant *Neisseria gonorrhoeae* (The Royal Australian College of Physicians, 2016). These organisms are major pathogens in both community and hospital settings and are increasingly resistant to major classes of antibiotics (Williamson & Heffernan, 2014). The Ministry of Health (2007a) identified that the most common mode of transmission of MDR organisms is through healthcare workers, either through contamination from an infected patient or through contaminated environmental surfaces. Organisms such as MRSA can survive for several months on certain surfaces (Neely & Maley, 2000). The importance of adhering to cleaning and disinfecting procedures is extremely important as a part of antimicrobial stewardship.

1.3.2 Farming and agriculture

There are several pathways for MDR bacteria to transmit through the environment. Collis et al. (2019) found that manure-contaminated pastures, animals, or animal-derived products from dairy farms are just some of the pathways through which resistant bacteria transmit. The risks associated with antibiotic use in meat production are well known, to the extent that changes in policies have been implemented in places throughout Europe and in New Zealand to reduce its impact (Heuer et al., 2011; Ministry of Health & Ministry for Primary Industries, 2017b). However, other sectors of intensive farming such as dairy farming have not been as well studied. Moderate correlations have been found between high intensity farming during winter and antibiotic resistant bacteria across twenty freshwater sites within the Southland region (Winkworth-Lawrence & Lange, 2016). In comparison to other countries, New Zealand is the 3rd lowest user of antibiotics in food animals, though this varies on a year-to-year basis and across different regions (Bryan & Hea, 2017; Hillerton et al., 2017).

The World Health Organisation has created a priority list for human pathogens that include ESBL-producing bacteria, which have been known to be associated with dairy cattle and the environment (Collis et al., 2019). Agriculture is one of the primary industries in New Zealand, with beef, sheep and dairy exports contributing over $23.8 billion towards the economy in 2020 (Statistics New Zealand, 2020b). In 2018, the Ministry for the Environment had the following data on land use in the South Island (Ministry for the Environment, 2018):

Region	Dairy farming (% of total land area)	Sheep & beef (% of total land area)	Other livestock (% of total land area)
Canterbury (including Banks Peninsula)	419,078 ha (9%)	1,723,270 ha (38%)	111,710 ha (2%)
Marlborough	18,366 ha (1%)	391,724 ha (31%)	6,758 ha (<1%)

Many recreational sites such as Department of Conservation (DoC) camping areas and walks have water sources derived from surface waters that run through agricultural areas. The high density of land used for farming increases the potential presence of MDR bacteria to which tourists and residents may be exposed.

1.3.3 Treated wastewater and irrigation

Treated wastewater has been found to be a source of antibiotic resistant bacteria. The use of this water on land releases resistance genes and antibiotic residues into the terrestrial environment (Gatica & Cytryn, 2013; Karkman et al., 2019). Antibiotic resistant bacteria in surface waters are frequently linked to wastewater treatment plants (Uyttendaele et al., 2015). Farkas et al. (2016) found that MDR bacteria were more prevalent in surface waters than in wastewater. They suggested that the bacteria were enriched through wastewater treatment before being released into surface waters.

In New Zealand, there are five ways in which treated wastewater is released into the environment (Ferguson, 2003): through

1) freshwater ecosystems (streams, lakes, and wetlands)

2) marine ecosystems (estuaries, harbours, and ocean)
3) land ecosystems (agricultural, horticultural, forestry and landscaped areas)
4) the atmosphere (i.e., wastewater aerosols)
5) landfills.

The use of wastewater as irrigation water is a method for conserving potable water. There are currently no legal guidelines in New Zealand to regulate antibiotic resistant bacteria in irrigation water, with even some untreated wastewater released to the environment through irrigation systems (Ferguson, 2003). In New Zealand, irrigation is predominantly used in pastoral farming. Between 2002 and 2019 there was a 91% increase in irrigated agricultural land, with largest amount of irrigated land found in Canterbury (Statistics New Zealand, 2021). In the Waimakariri district, a large proportion of irrigation water is sourced from the Waimakariri river (personal communication Waimakariri District Council), a river which has increasingly become contaminated with antibiotic resistant *E. coli* (Schousboe et al., 2015). Without screening for antibiotic resistant organisms, treated and untreated wastewater being released into surface waters creates a pathway for exposures to antibiotic resistant organisms. The quality of these surface waters becomes especially important when they are sourced as drinking and food preparation water. Further, the use of water for irrigation which may harbour antibiotic resistant bacteria may aid in the spread and dissemination of resistant bacteria and resistant determinants.

1.4 Objectives of this study and hypotheses

My area of research is on human exposure to water with antibiotic resistant bacteria. The objective of this study was to determine the baseline numbers of antibiotic resistant bacteria in surface freshwater that is used for drinking and food preparation. The scope was concentrated on exposures that would be common for tourists. In part, this was meant to draw attention to the difference in potential exposures of tourists and the general domestic population. However, my research was not limited to the tourist experience but included local communities near to tourist hotspots.

Tourism was one of New Zealand's largest sectors prior to the COVID-19 pandemic, with international and domestic tourism contributing $40.9 billion to New Zealand's economy

annually (Tourism New Zealand, 2020). Many tourists arrive by plane, transit through airports, stay in DoC campsites or visit areas where surface water is used for cooking, drinking, and swimming. The work begins at the first point of their entry, airplane water, and follows along some popular locations around the South Island. The bacterium *E. coli* was chosen as an indicator organism because it is a useful surrogate indicator of faecal presence and disease-causing organisms in an environment and *E. coli* levels can be applied to existing guidelines as an indicator of human health risk.

I believe the implications of this research are important as the increase in AMR affects New Zealand in multiple ways. Most drinking water in New Zealand is tested but there is a significant proportion of water sources that remain untested and unregulated (Ministry of Health, 2019a). Along with this, even though current water monitoring reports *E. coli* levels, it does not include screening for antibiotic resistance. There are often warning signs to boil water before consumption at sites where the water quality is poor, however, tourists often still wash their hands and brush their teeth with the contaminated water, which allows for alternate pathways of transmission into the human body.

1.4.1 Main hypotheses

Hypothesis 1: Water from the water storage tanks of long-haul international flights will have a higher frequency of MDR *E. coli* than domestic flights within New Zealand. This hypothesis would be supported if MDR *E. coli* were detected in the water. This hypothesis would not be supported if there were no difference in MDR *E. coli* concentrations.

Hypothesis 2: Tourists will be exposed to more MDR *E. coli* than recognised because of their concentrated experience with water supplies that are rarely or never monitored. This was tested by measuring the concentration of both *E. coli* and MDR *E. coli* in these water sources. This hypothesis would be supported if MDR *E. coli* were detected in the water. This hypothesis would not be supported if MDR *E. coli* levels were comparable to water supplied and monitored by municipalities.

Hypothesis 3: MDR *E. coli* isolated from the environment will be able to share resistant determinants with susceptible bacteria through horizontal gene transfer. This hypothesis will be

supported if resistance is observed in a susceptible lab strain after conjugation experiments. This hypothesis would not be supported if sharing of resistance genes is not observed.

1.4.2 Objectives

1. Isolate *E. coli* and antibiotic resistant *E. coli* from selected surface freshwater that is sourced as drinking water across the Marlborough Sounds, Banks Peninsula and Wider Canterbury Area using a phenotypic culture-based screening method.
2. Determine frequency of resistance to eight clinically significant antibiotics using *E. coli* isolates and observing for ESBL-production.
3. Determine the minimum inhibitory concentration (MIC) of *E. coli* isolates.
4. Quantification and comparison of patterns of resistance across different regions. This information will allow me to observe whether land use patterns can help to predict resistance.
5. Determine the frequency of resistance genes being carried by conjugative plasmids.
6. Evaluate the reliability of phenotyping and genotyping using sequenced isolates.

The study emphasizes geographic range over replication depth. This is because I was trying to get an overview of what is present in the water in different locations. Areas that had high *E. coli* counts and MDR *E. coli* were considered ‘areas of interest’ and were sampled from seasonally to observe any changes. Though this research has a broad overview, the primary focus was on surface waters sourced as drinking water.

1.5 Thesis Organization

Chapter 2 reports on the water quality data from each sampling location, including total *E. coli,* total *E. coli* resistant to three antibiotics, and total mesophilic bacteria. Chapter 3 investigates the resistance profiles of the *E. coli* isolates, as well as observing for the presence of ESBL-producing *E. coli* and conjugative donors. Finally, Chapter 4 discusses the underlying genetic determinants that confer resistance for 20 isolates.

Chapter 2: Water quality in the Banks Peninsula, Marlborough and Canterbury regions

Water is both a collector and distributor of microbes. As a collector, it is a like a library with individual genotypes representing the outcomes of evolutionary processes in a way similar to how books preserve the history of literary thought and record. Water at times can lend members of its collection out to us and other water users. Linking the water to the water user through the common thread of what microbes they share provides an important framework for my analysis of multidrug resistant bacteria in the environment. For my research, the focus is only on the species *E. coli* which is used by me and many others as an indicator of water quality. The additional dimension of my work is to also use it as an indicator of antibiotic resistance in the environment. Resistant bacteria and resistance genes are ubiquitous in the environment. My focus on *E. coli* is because of my interests are in the increase of number and distribution of these genes. *E. coli* can acquire resistance genes from various pathways in the environment, and these resistance determinants can be transferred to human pathogens. Only since the adoption of antibiotics by people, and the change in industrial capability to discover, modify and commercialise them around the middle of the 20th century, have these genes begun to colonise species such as the commensals and pathogens of people. That is why *E. coli* is a good indicator bacterium for both water quality and antibiotic resistance.

In this chapter, I follow the hypothetical journey of a tourist in New Zealand who has arrived on an airplane. The microbiological risk from airplane storage tank water from long haul and short haul flights are compared. International long haul flights are the first entry point for tourists visiting New Zealand, and while they are here, they may catch domestic flights to travel around. Next, I follow the tourist who wants to explore the country's spectacular environment to sites in rural areas with different sources of drinking water. Antibiotic resistant bacteria from these sources are also compared. Water quality data from sites across the Banks Peninsula, Marlborough, and Wider Canterbury area (herein referred to as Canterbury) are used to determine whether different regions may have different water quality and try to identify causes. Finally, I will present a deeper exploration of the water quality results from a case study

observing whether there are seasonal effects on the sizes and densities of *E. coli* and antibiotic resistant *E. coli* in the rural town of Okains Bay.

2.1 Airplane water

The water storage tanks in airplanes potentially harbour multidrug resistant bacteria. The supply chain journey from the water source to the water service vehicle to the airplane water storage tank can be an enriching environment for bacteria. A 2003 study by the Association of Port Health Authorities and the Public Health Laboratory Service in the United Kingdom found that coliform contamination in airplane storage tanks was strongly associated with the supply chain (Nichols, 2003). The water tanks on planes are rarely emptied or cleaned; they are only emptied when the water on board has been entirely consumed or when the aircraft is not in operation (Handschuh et al., 2015). According to media sources, in 2004 the United States Environmental Protection Agency found that 15% of samples from 300 planes contained coliforms (Gajanan, 2017). This becomes a larger issue when the quality of the water source is poor. Nichols (2003) showed that variation in coliform contamination was associated with particular airports, indicating poor water quality from the sources. For example, the well at Christchurch International Airport has a microbial water quality grade of ‘D’, which means that it has an unsatisfactory level of risk for use as potable water (Institute of Environmental Science and Research, 2021). If this water is used to refill the water storage tanks in airplanes, it could present further risk of exposure to harmful bacteria that are antibiotic resistant. Because this is the main passage for most tourists into our country, it is important to investigate the risks that may stem from these exposures. Though water on airplanes is not commonly used for drinking water, there are other pathways that people interact with this water that exposes them to risk of infection by antibiotic resistant organisms (Figure 2).

Exposure	Pathway	Risk of infection by antibiotic resistant organisms
	Ingestion	Gastrointestinal infection
	Ingestion Open wounds	Gastrointestinal infection Skin infection
	Ingestion Open wounds	Gastrointestinal infection Skin infection
	Ingestion	Gastrointestinal infection
	Ingestion Open wounds Transmission through body openings	Gastrointestinal infection Skin infection Bladder infections

Figure 2 Risk analysis of exposure, pathways, and infection from antibiotic resistant organisms.

2.2 Surface waters that are sources of drinking water

Water recreational activities are an important part of the local culture and lifestyle in New Zealand, and prior to the COVID-19 pandemic, were significant also to tourists. Such activities are concentrated in the summer months when access to potable water is particularly important. Potable, recreational, and grey water must all meet different standards (Table 1). In New Zealand, all potable water must meet the minimum quality standards for drinking water as outlined by the Ministry of Health and the New Zealand Drinking Water Association (Ministry of Health, 2018). The Drinking Water Standards for New Zealand (DWSNZ) outlines the maximum acceptable value for *E. coli* is less than 1 in 100 mL of sample for drinking water (Ministry of Health, 2018). Most water supplies are monitored, though even not all of these must be reported (Institute of Environmental Science and Research, 2021). Water quality monitoring, especially in rural areas, helps to identify causes of contamination, and regular monitoring provides a better picture of where potential sources of contamination may be (Phiri et al., 2021).

Table 1 Water quality required for different household activities. Table sourced from Ministry of Health (2007b)**.**

Type of use	Quality requirements
Drinking, cooking, food preparation	Biologically and chemically safe
Bathroom	Biologically safe, chemically safe for skin contact
Laundry, toilet flushing	Should not cause stains or damage clothing
Outdoor (eg, irrigation, car washing)	No special requirements but safe for skin contact

Surface waters are commonly used for drinking water in New Zealand. If they are distributed in a distribution zone, then treatment occurs at the plant to ensure the potable water is compliant with the DWSNZ. However, the DWSNZ does not require compliance for private distributions or reticulated town supplies for fewer than 1,500 person days (ie., 25 people for fewer than 60 days) (Ministry of Health, 2017). Surface waters in rural areas where campgrounds and small rural communities are based are likely to fall under these categories. These places are generally affected by human activity, and the surface waters are highly likely to be contaminated by animal waste, sewage effluent and agricultural fertilisers (Ministry of Health, 2017). Rivers and streams, which are some of the surface sources which my thesis focuses on, often have poor water quality, and may be contaminated by wildlife, farm animals, decaying vegetation, algae, wastewater, and other human inputs (Ministry of Health, 2007b). Table 2 describes the common causes of contamination in surface waters and their likely sources. The Ministry of Health suggests treating noncompliant raw water with chlorine dosing, ultraviolet (UV) light treatment, boiling or chemical treatment in order to meet the minimum requirements for safe drinking water (Ministry of Health, 2007b).

Table 2 Common causes of contamination in surface waters and their sources. Sourced from Ministry of Health (2017).

Cause	Problem	Likely Source
Bacteria	Waterborne disease	Human and animal wastes
Nitrate	Bottle fed infants can have breathing problems (blue baby syndrome)	Fertilisers, sewage, animal effluent, clover pasture
pH	When less than 6.5, corrosion of plumbing materials, possibly causing copper or lead to be dissolved into the water	Soft water, CO2 rich groundwaters
	When greater than 8.5, scale formation in hot water cylinders and on heating elements causing reduced efficiency and premature failure. Also, can cause excessive scale build-up in pipes	Many groundwaters
Protozoa	Waterborne disease	Human and animal wastes
Turbidity	Appearance, and interference with disinfection	Suspended particles of natural and human or animal origin
Viruses	Waterborne disease	Human and animal wastes

Phiri et al. (2021) performed a survey of 15 public DoC campgrounds in New Zealand. They measured *E. coli* as an indicator of drinking water quality. The sites were supplied by water collected from roofs or surface water for their drinking water, and a mixture of water quality treatments from none to UV irradiation, filter filtration, chemical treatment, or a combination of the last three. In more than 50% of sampling occasions, the drinking water was not compliant with the national standards. Their findings highlighted the importance of proper water treatment, as the use of water filters alone was not sufficient to provide safe drinking water. Rather, the use of UV treatment alone, or in conjunction with chemical treatment, was necessary to provide potable water.

Users must be warned when the water available at these campsites and outdoor recreational areas do not meet the DWSNZ minimum requirements. This is generally done through a 'Boil Water Notice' sign. This sign warns users to boil the water for at least three minutes before consuming to minimize the potential risk posed by the contaminated water. The risk of infection by an antibiotic resistant organism arises from exposure to water contaminated with them. Even if the water is not immediately safe to drink, it could be used for hygiene purposes such as showering, hand washing and even cleaning teeth. Access through ingestion or through open wounds can lead to serious infections (Figure 2). As antibiotic resistance in the New Zealand environment grows, so does the risk of being infected by an antibiotic resistant organism.

2.3 Case study: Okains Bay

Okains Bay was selected as a case study for my thesis because it is a good example of a small rural community that sources their drinking water from a surface water that is non-compliant with DWSNZ. Okains Bay is a rural town in the Banks Peninsula area in the South Island. It has a population of approximately 105 people (Institute of Environmental Science and Research, 2021). Around 100 residents (40 households), and the Christchurch City Council-owned campground, are involved in a private scheme organized by the Okains Bay Water Committee to source drinking water from the nearby Opara Stream (Law, 2018). The Opara Stream, however, does not meet the DWSNZ, and requires at least boiling before it is considered potable (Institute of Environmental Science and Research, 2021). Other residents' source their drinking water from nearby tributaries that feed into the Opara Stream, or from the aquifer that is the source of the Opara Stream (personal communication with residents). Many of the residents fill up large containers of clean drinking water from Akaroa or Christchurch or buy bottled water (Law, 2017). Residents pay $30 a year for maintenance, while the Okains Bay School spends over $1,000 a year for water quality testing and filtration systems to provide the school children and the staff safe drinking water (Law, 2017). However, the filtration systems at the schoolhouse does not consistently provide clean water, leaving the staff and children exposed to untreated water (private communication with the school's principal). In 2020, the school moved into a new building, with updated filtration systems. Through communications with the principle, I was informed that the treatment systems at the new school were sufficient for the drinking water to be compliant with DWSNZ standards. Okains Bay is a catchment area, and the water quality is

influenced by rainfall. Faecal contamination of the Opara stream can occur from the runoff from land grazed sheep, cattle, septic tank seepage, and even from runoff from roofs (Bolton-Ritche, 2008).

2.4 Main hypotheses

Hypothesis 1: Water from the water storage tanks of long-haul international flights will have a higher frequency of *E. coli* than domestic flights within New Zealand.

Hypothesis 2: Antibiotic resistant *E. coli* will be found in areas with water supplies that are rarely or never monitored but are high in visitation by tourists. This was tested by measuring the concentration of both *E. coli* and resistant *E. coli* in these water sources.

Hypothesis 3: *E. coli* concentrations will be predictive of resistant *E. coli.*

My first hypothesis was tested by observing the water quality of airplane water, using *E. coli* as an indicator, and comparing the *E. coli* concentrations between short domestic flights and long-haul flights. My second hypothesis was tested by observing the quality of drinking water across the Canterbury, Banks Peninsula and Marlborough regions using *E. coli* as an indicator and comparing the antibiotic resistant *E. coli* and mesophile profiles between sites. Sites were selected based on their source of drinking water. My final hypothesis will be tested by observing whether there is a correlation between *E. coli* concentrations and resistant *E. coli* concentrations.

Methods

2.5 Sampling Locations: Airplane Water

Airplane water samples were gathered when opportunities to do so arose. Samples were obtained from volunteers among acquaintances and family members who were traveling on either domestic or international flights. Table 3 details a list of the flights and the airlines.

Table 3 Departure and arrival locations for airplanes included in the water quality study.

Departing Location	Arrival Location	Airline	Date
Fiji	NZ	Air New Zealand	8th May 2019
Abu Dhabi	Sydney	Emirates	5th April 2019
Sydney	Christchurch	Emirates	5th April 2019
Rarotonga	Auckland	Air New Zealand	17th May 2019
Rotorua	Christchurch	Air New Zealand	20th April 2019
Dunedin	Christchurch	Air New Zealand	2nd June 2019
Auckland	Christchurch	Air New Zealand	21st July 2019
Auckland	Christchurch	Jetstar	20th October 2019

2.6 Sampling Locations: Drinking Water Samples

Samples were obtained from selected rural campgrounds and outdoor recreational areas in the Canterbury, Banks Peninsula and Marlborough regions. These areas were chosen because they have a high volume of tourist traffic and it is common for fresh surface water to be sourced as drinking water. Specific locations were chosen through advice from the Renwick Department of Conservation (Renwick DoC), Councillor Lan Pham from Environment Canterbury (ECan) and through personal investigation. Drinking water samples were split into three categories: Treated vs Stream vs Other. The category Treated includes water that is sourced from surface waters but is treated locally to provide potable water for users. The category Stream represent sites where the drinking water is sourced untreated from streams. The category 'Other' includes untreated water sources such as tributaries, rivers, and springs which were included less frequently in my

samples in comparison to Stream samples. The following describes the sampling locations within each region.

2.6.1 Canterbury

Sampling Location	Area	Water supply	Number of times sampled
Chamberlain Ford Recreation Reserve	Leeston	Water supply not suitable for drinking (Selwyn District Council).	1
Coes Ford Camping Ground	Springston		1
Timber Yard Point Lakeside Domain Camping Area	Leeston		1
Omihi Reserve Campground	Kaikoura	Boil water notice. Drinking water comes from a stream which runs through Goose Bay Camp. It is diverted to a pump and filtration facility for distribution to all camps. The water is tested regularly, and its current status is that it is safe to drink (personal communication with Kaikoura Information and Tourism).	1
Paia Point Campground			1
Rakaia Huts Camping Ground	Rakaia	Town reticulated supply.	1
Sharplin Falls Carpark	Mount Somers	Sourced from stream. All drinking water in this area should be sterilised, filtered or boiled for 3 minutes before use (Department of Conservation, 2014).	1

2.6.2 Banks Peninsula

Sampling Location	Area	Water supply	Number of times sampled
Little River Campground	Okuti Valley	Treated through a private system. Water sourced from Okuti Valley Stream.	1
Okuti Valley River		Untreated. This is the source of drinking water for the campground.	1
Okana River and Police Creek	Little River	These are the sources of drinking water for Little River. Water is treated before distribution.	1
Okains Bay	Banks Peninsula	I had seven sampling sites in the Okains Bay area. The approximate location of each site can be found in Figure 3.	4 (Seasonal sampling)
Site 1: Upstream		This site is approximately 5.3 km upstream of the estuary. It runs through a farm parallel to Okains Bay Road	
Site 2: Tributary		This site is approximately 5.3 km upstream of the estuary. This tributary travels under the Okains Bay Road and feeds into the 'Upstream' site.	
Site 3: Whitebait Bridge		This site is approximately 2.8 km upstream of the estuary. This bridge connects Okains Bay Road to Okains River Road and Schoolhouse Road. The Opara Stream runs parallel to Okains Bay Road approximately 1.5m away from the road. At this site, the Opara Stream is flowing through the township.	
Site 4: Okains Bay School		This site is approximately 2 km upstream of the estuary. The sample was taken from the kitchen tap in the school.	
Site 5: Okains Bay Campground Kitchen		This site is approximately 0.25 km upstream of the estuary. This is one of two kitchens at the Okains Bay Campground, however, the other kitchen was closed for maintenance during sampling.	
Site 6: Estuary		This site is where the Opara Stream meets the South Pacific Ocean. The estuary has open access to campers and visitors.	
Site 7: Residential Home		The drinking water from this residential home is sourced from a tributary that feeds into the Opara Stream. The water samples were obtained from the kitchen tap in their home.	

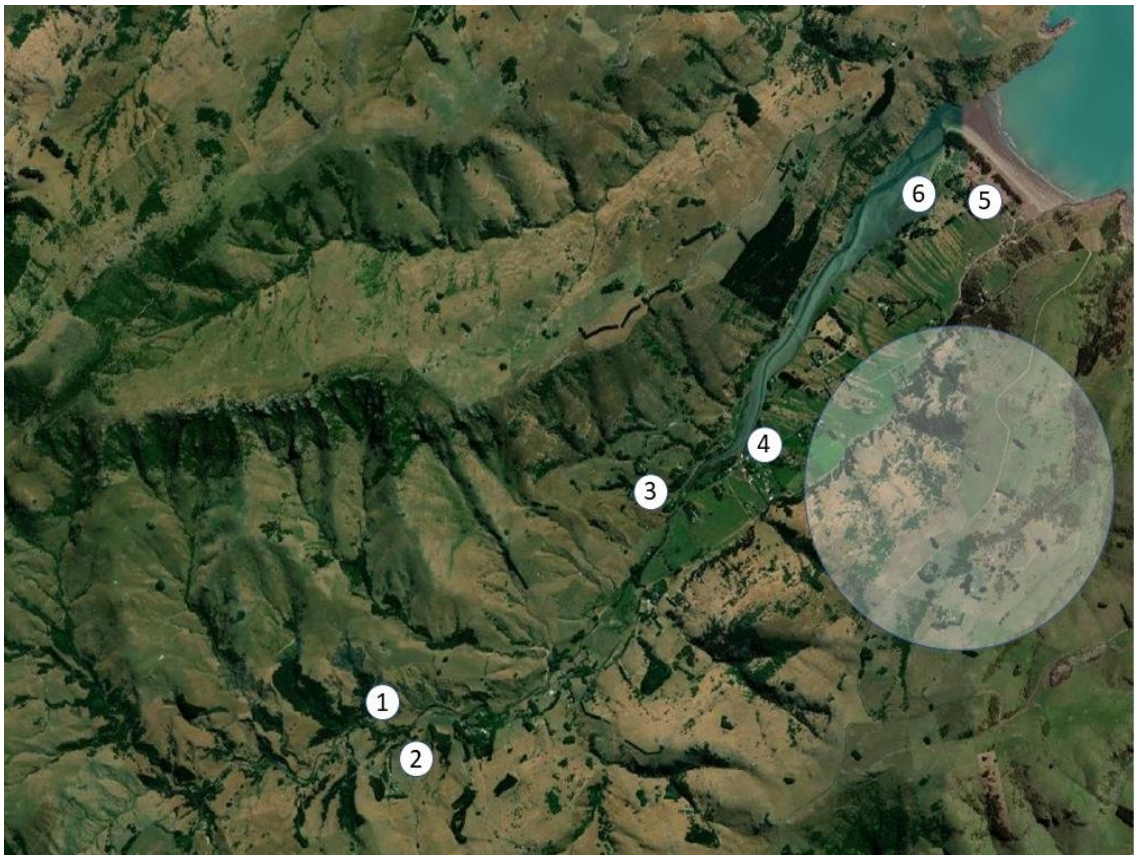


Figure 3 Sampling sites from Okains Bay: 1. Upstream; 2. Tributary; 3. Whitebait Bridge; 4. Okains Bay School; 5. Camp Kitchen; 6. Estuary; Large opaque circle indicates area of the residential home.

2.6.3 Marlborough

Sampling Location	Area	Water supply	Number of times sampled
Pukatea/ Whites Bay campground	Waikawa	Boil water notice. Water sourced from Pukatea awa/stream. Information obtained from Renwick DoC.	2
Rarangi campground	Rarangi	Water supplied by North Rarangi Water Supply Inc, reticulated town supply. Information obtained from Renwick DoC.	2
Marfells Beach campground	Lake Grassmere	Boil water notice. Water supplied by Marlborough District Council from the Black Birch River water supply for the lower Awatere Valley. Information obtained from Renwick DoC.	2
Whatamango Bay Campground	Queen Charlotte Sound/Tōtaranui	Boil water notice. Spring water source (Phiri et al., 2021).	3

2.7 Sampling Methods

2.7.1 Airplane Water

At least 100 mL of water was collected from the sink in the airplane toilet. Where possible, samples were kept cool (at ambient cabin temperature) and were processed as soon as they arrived. For domestic flights, 50 mL sterile falcon tubes were used for water collection. For long haul flights, unopened bottles of water were used, and the seal was broken, and water emptied prior to sample collection to minimize contamination.

2.7.2 Drinking Water

1. Personal Collection

At least 800 mL of water was collected in 1 L sampling bottles from each sampling site, with 3 replicates for each location. The bottle was only opened immediately prior to gathering the sample to maintain the sterility of the bottle. The temperature and pH of the water was also taken.

2. Samples by courier

At least 800 mL of water was collected in 1 L sampling bottles from each sampling site with 3 replicates for each location. Sample gatherers were advised to wear gloves and only open the bottle immediately prior to gathering the sample to maintain the sterility of the bottle. They were instructed to record the time the sample was taken (and temperature if possible) and to send the samples on the same day in a freezer box with ice packs.

2.8 Culturing methods

2.8.1 Airplane Water

For airplane water samples, either 50 mL or 100 mL was filtered and placed onto TBX (Tryptone Bile Glucuronic agar; Himedia) only. 100 µL was spread plated onto R2A (Reasoner's 2A agar; Difco). Plates were incubated 18 h with plates selective for *E. coli* incubated at 44°C and plates permissive for mesophilic bacteria incubated at 30°C.

2.8.2 Drinking Water

100 mL of the samples were filtered through Büchner funnels onto 0.45 µm nitrocellulose filters (Merck Millipore) and the filters were placed onto TBX, TBX supplemented with ampicillin (8 µg mL^{-1}), ciprofloxacin (1 µg mL^{-1}) and chloramphenicol (8 µg mL^{-1}). Along with the four plates, 1 ml of the sample was spread plated onto TBX, and 100 µL onto R2A for observation of mesophilic bacteria. Plates were incubated 18 h with plates selecting for *E. coli* incubated at 44°C and plates permissive for mesophilic bacteria incubated at 30°C.

2.9 Toothbrush experiments

I was interested in observing whether *E. coli* in the water could survive on toothbrush heads. To test this, an exploratory study was conducted investigating three different methods of water exposure, and the differences between using toothpaste and not using toothpaste (Table 4). Full immersion involved soaking the toothbrush head in the water completely, aerosol involved spraying the water on the toothbrush head and consistent flow involved pouring water over the toothbrush head to mimic water coming out of a tap. 50 mL of water sampled from Okains Bay Campground Kitchen was used for each condition, and sterilized distilled water was used as a negative control. The initial *E. coli* concentration in the water from Okains Bay Campground Kitchen was determined to be 8×10^{1} cfu mL^{-1}. Each condition was tested in triplicates and repeated with dilutions up to 1×10^{-2} cfu mL^{-1}. After incubation, the toothbrush heads were immersed in 25 mL of sterilized water and vortexed for 30 sec, sonicated for 5 min, and vortexed for 30 sec again to detach any *E. coli*. 20 mL was filtered onto TBX and 1 mL was spread plated onto TBX. Immersion in Luria-Bertani (LB; Invitrogen) agar was employed to observe *E. coli* on the toothbrush head. This study was based on Kobayashi et al. (2009) with modifications.

Table 4 Different methods of water exposure on toothbrushes with and without toothpaste.

Method	**Condition**	**Time (h)**
Full immersion	Toothpaste	18
	No toothpaste	18
Aerosol	Nil	18
Consistent flow	Toothpaste	3
	No toothpaste	3
	Toothpaste	6
	No toothpaste	6
	Toothpaste	18
	No toothpaste	18

2.10 Statistical analysis

R was used for all statistical analyses in this thesis (R Core Team, 2020). Plots of residuals were used to check for violations of assumptions in all analyses. In all cases, the assumption of normally distributed residuals was violated, even after log transformation. To handle this, generalized linear models (GLM) and generalized linear mixed models (GLMM) were employed. Distribution of residuals from the models were investigated to observe whether the residual structure fit the assumptions made in the model.

2.10.1 Airplane water

For the airplane storage tank water, I was interested in the difference in microbial load between long haul and domestic flights, and whether there were differences between the airlines. In total, eight flights were included in the analysis (four long haul, four domestic) from three different airlines; Air New Zealand (ANZ), Emirates and Jetstar. A generalized linear model (GLM) with a Poisson log link was chosen as the mesophilic bacteria counts followed a Poisson distribution, and residuals were not normally distributed.

2.10.2 Drinking water

Across the three regions, I was interested in whether *E. coli* abundance and resistant *E. coli* abundance was dependent on the water source, and whether there were differences across the regions. In total, 12 sites with 3 replicates per site across the Banks Peninsula, Marlborough and Canterbury region were chosen for analysis (Table 5). Counts of mesophilic bacteria were always above the detection limit. A generalized linear mixed model (GLMM) with a Poisson log link was chosen as the *E. coli* per 100 mL counts followed a Poisson distribution, the residuals were not normally distributed, and the water sources categories were unbalanced. Over dispersion was accounted for by assigning each observation a unique value and including this as a random effect in the analysis.

A GLMM with a Poisson log link model was selected for the ampicillin resistant *E.* coli populations, for the same reasons as the analysis for the total *E. coli* counts per 100 mL. Over dispersion was accounted for by assigning each observation a unique value and including this as

a random effect in the analysis. For *E. coli* resistant to chloramphenicol or ciprofloxacin, often counts were below the detection limit. Due to this, a GLMM with a binomial link was selected.

I was interested in whether the total *E. coli* counts per 100 mL could be predictive of resistant *E. coli* populations. To determine this, a linear regression was performed on log transformed total *E.* coli counts and resistant *E. coli* counts. This analysis was limited to ampicillin resistant populations as ciprofloxacin resistant and chloramphenicol resistant counts were often below the detection limit. A small constant of 0.0001 was added prior to log transformation, and observations where *E. coli* counts were below the detection limit were omitted from the analysis.

Table 5 Sites included in the drinking water GLMM analysis.

Location	Site	Water source
Banks Peninsula	Residential Okains August	Other
	Okains Campground Kitchen	Stream
	Okains School	Stream
	Little River Campground	Treated
Marlborough	Marfells Beach Campsite	Other
	Whatamango Bay Campground	Other
	Whites Bay Campground	Stream
	Rarangi campground	Treated
Canterbury	Lakeside Domain	Other
	Sharplin Falls	Stream
	Omihi Campground	Treated
	Paia Point Campground	Treated

2.10.3 Okains Bay

Across all six sites, I was interested in whether *E. coli*, resistant *E. coli* and mesophilic bacteria abundance was dependent on the site, and whether there were differences across four seasons. All 6 sites across four seasons were included in the analysis, except for one site (Okains Bay site 1, tributary) in autumn as the tributary was too low to obtain an adequate sample. This sample was omitted form the analysis. Counts of mesophilic bacteria were always above the detection limit. To compare differences between seasons, a GLMM with a Poisson log link was chosen for the *E. coli* analysis, the ampicillin resistant *E. coli* analysis and the mesophilic bacteria analysis as the bacterial counts followed a Poisson distribution, the variance was not normally distributed,

and the categories were unbalanced. Over dispersion was accounted for by assigning each observation a unique value and including this as a random effect in the analysis. For *E. coli* resistant to chloramphenicol or ciprofloxacin, often counts were below the detection limit. Due to this, a GLMM with a binomial link was selected.

To compare differences between sites in each season, a GLM with a Poisson log link was selected as was chosen for the *E. coli*, the ampicillin resistant *E. coli*, the chloramphenicol resistant *E. coli* and the ciprofloxacin resistant *E. coli* analysis *as* the *E. coli* per 100 mL counts followed a Poisson distribution and the variance was not normally distributed. Chloramphenicol resistant *E. coli* were below the detection limit in all seasons except spring, therefore the analysis was only done for this season. Similarly, ciprofloxacin resistant *E. coli* were below the detection limit in all seasons except autumn, therefore the analysis was only done for this season.

Results

2.11 Airplane Water

Mesophilic bacteria were detected at 10^3 cfu mL^{-1}. *E. coli* was always below the detection limit. Long haul flights had significantly more mesophilic bacteria than domestic flights ($p < 0.0001$). ANZ and Emirates both had significantly more mesophilic bacteria than Jetstar ($p < 0.0001$). Details from the statistical analysis can be found in Appendix A1.

2.12 Water Quality Results: Total *E. coli* and mesophilic bacteria

A summary of the water quality results can be found at the end of this section in Table 6. Mesophilic bacteria were detected at 10^2 to 10^4 cfu mL^{-1}. Treated had significantly lower mesophilic bacterial numbers than Stream and Other ($p < 0.0001$). Banks Peninsula had significantly more mesophilic bacteria than Canterbury ($p < 0.0001$), and Canterbury had significantly higher mesophilic loads than Marlborough ($p < 0.0001$).

The total *E. coli* count varied between 10^0 and 5×10^2 cfu 100 mL^{-1} across the three conditions. There was a significant difference between Treated and Stream ($p = 0.001$), and no significant differences between Treated and Other ($p = 0.053$) or Stream and Other ($p = 0.3940$). There were no significant differences in *E. coli* counts between regions, however, within Canterbury, there was significantly more *E. coli* in Stream than in Treated ($p < 0.046$; Appendix A2). The highest *E. coli* count was detected at Site 11 in Canterbury (source = Stream), while the lowest *E. coli* counts were detected in both Other and Treated water sources across the three regions. Figure 4 displays the distribution of *E. coli* across the water sources and regions.

Boxplot of *E. coli* distribution across the water sources and regions

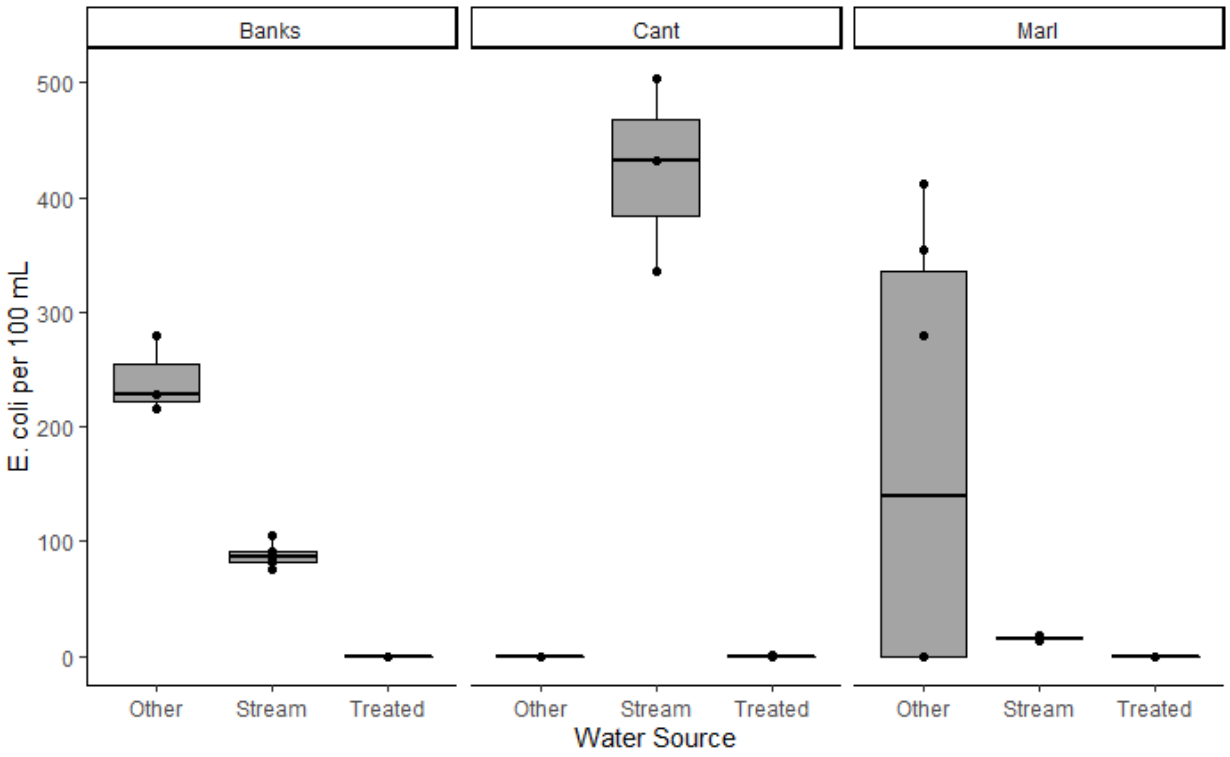


Figure 4 Distribution of *E. coli* across Other, Stream and Treated across the three regions. Banks = Banks Peninsula; Cant = Canterbury; Marl = Marlborough Sounds.

2.13 Distribution of ampicillin resistant populations across water sources

There were significant differences in ampicillin resistant *E. coli* concentrations between Treated Stream ($p < 0.0184$). Other in Banks Peninsula had significantly higher numbers of ampicillin resistant *E. coli* than Treated in Canterbury ($p = 0.0027$). Within Canterbury, there was significantly more resistant *E. coli* in Stream than in Treated ($p < 0.0064$; Appendix A3).

2.13.1 Are *E. coli* population densities predictive of antibiotic resistant population densities?

Following from Van Hamelsveld et al. (2019) I wanted to investigate whether total *E. coli* counts could be predictive of resistant populations in the environment. Ampicillin resistant populations were selected for this analysis. There was no association between total *E. coli* counts per 100 mL and ampicillin resistant populations ($R^2 = 0.0385$; Figure 5).

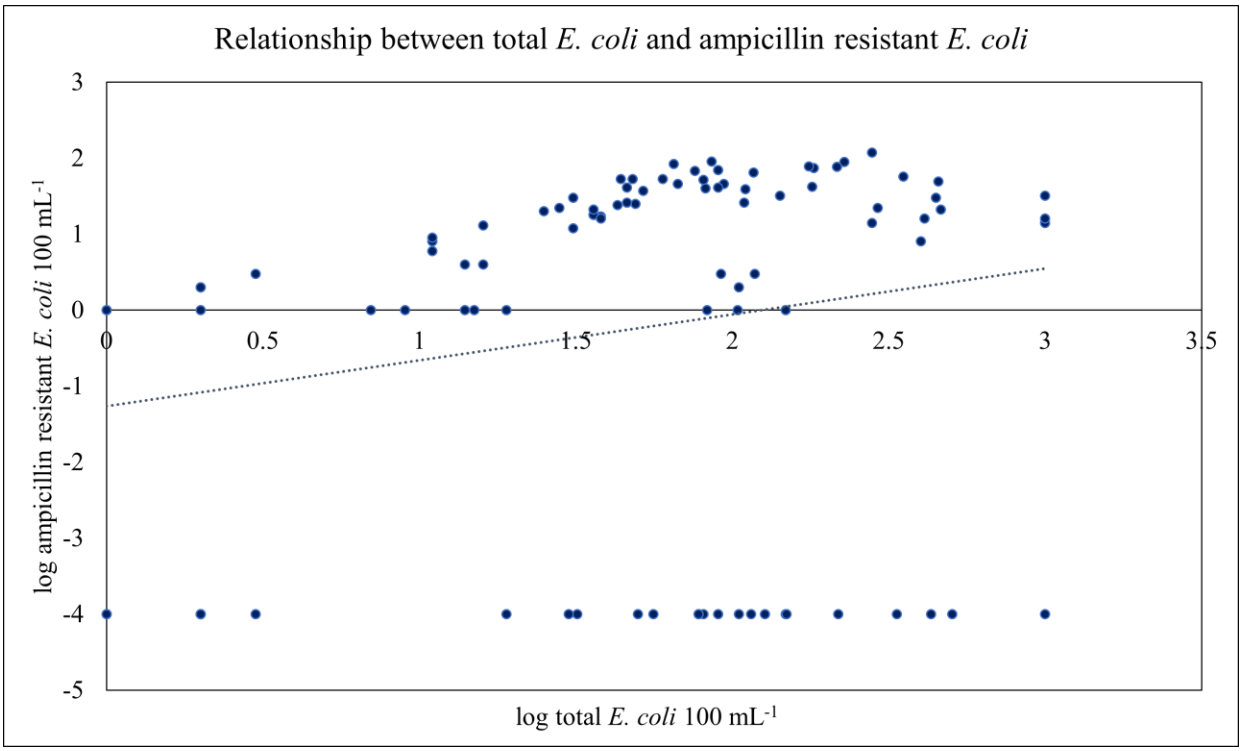


Figure 5 The relationship between total *E. coli* and ampicillin resistant *E. coli* per 100 mL. No correlation was found ($R^2 = 0.0385$).

2.14 Distribution of chloramphenicol resistant and ciprofloxacin resistant populations across water sources

There were no significant differences in chloramphenicol resistant *E. coli* populations across the three water sources or the three regions (Appendix A5). There were no significant differences in ciprofloxacin resistant *E. coli* populations across the three water sources or the regions (Appendix A4). Of the sites included in this analysis, chloramphenicol resistant and ciprofloxacin resistant *E. coli* populations were only detected on two sampling occasions, and in one instance, ciprofloxacin resistance was just above the detection limit.

Table 6 Summary of results (p values) from the water quality analysis. Comparisons between water sources, and between regions. * = significant difference; Amp^R = ampicillin resistant; Chl^R = chloramphenicol resistant; Cip^R = ciprofloxacin resistant; BP = Banks Peninsula; C = Canterbury; M = Marlborough.

Comparisons	Total *E. coli*	Amp^R *E. coli*	Chl^R *E. coli*	Cip^R *E. coli*	Mesophilic bacteria
Other – Stream	0.3940	0.6062	1	1	0.7444
Other – Treated	0.0534	0.1380	1	1	<0.0001*
Treated - Stream	0.0016*	0.0184*	1	1	<0.0001*
BP – C	0.4670	0.1523	1	1	<0.0001*
BP – M	0.6850	0.0986	1	1	0.0930
C - M	0.9276	0.1244	1	1	<0.0001*

2.15 Okains Bay

2.15.1 Seasonal effects

Mesophilic bacteria were detected at 10^0 to 10^3 cfu mL^{-1} and varied significantly between seasons ($p < 0.0001$). Summer samples had significantly more mesophilic bacteria than the other seasons ($p < 2e^{-16}$). Figure 6 compares the distribution of *E. coli* and resistant *E. coli* across the six sites and four seasons. *E. coli* concentrations in the water were 10^0 to 10^3 cfu mL^{-1}. Post hoc analysis revealed significant differences between winter and autumn ($p = 0.0242$) and autumn and summer ($p < 0.0001$). Overall, the highest *E. coli* concentrations were detected in summer. Detailed statistical outputs can be found in Appendix A6.

Ampicillin resistant *E. coli* concentrations in the water were between 10^0 and 10^2 cfu 100 mL^{-1} and varied significantly between seasons ($p < 2e^{16}$). Post hoc analysis revealed significant differences between winter and autumn ($p = 0.242$), and autumn and summer ($p < 0.0001$). Ampicillin resistant populations were detected in all seasons except autumn, and concentrations varied across sites. Detailed statistical outputs can be found in Appendix A7. Chloramphenicol resistant *E. coli* was below the detection limit in all seasons except spring, however, this detection was not significant. Ciprofloxacin resistant *E. coli* was below the detection limit in all seasons except autumn, however, this detection was not significant.

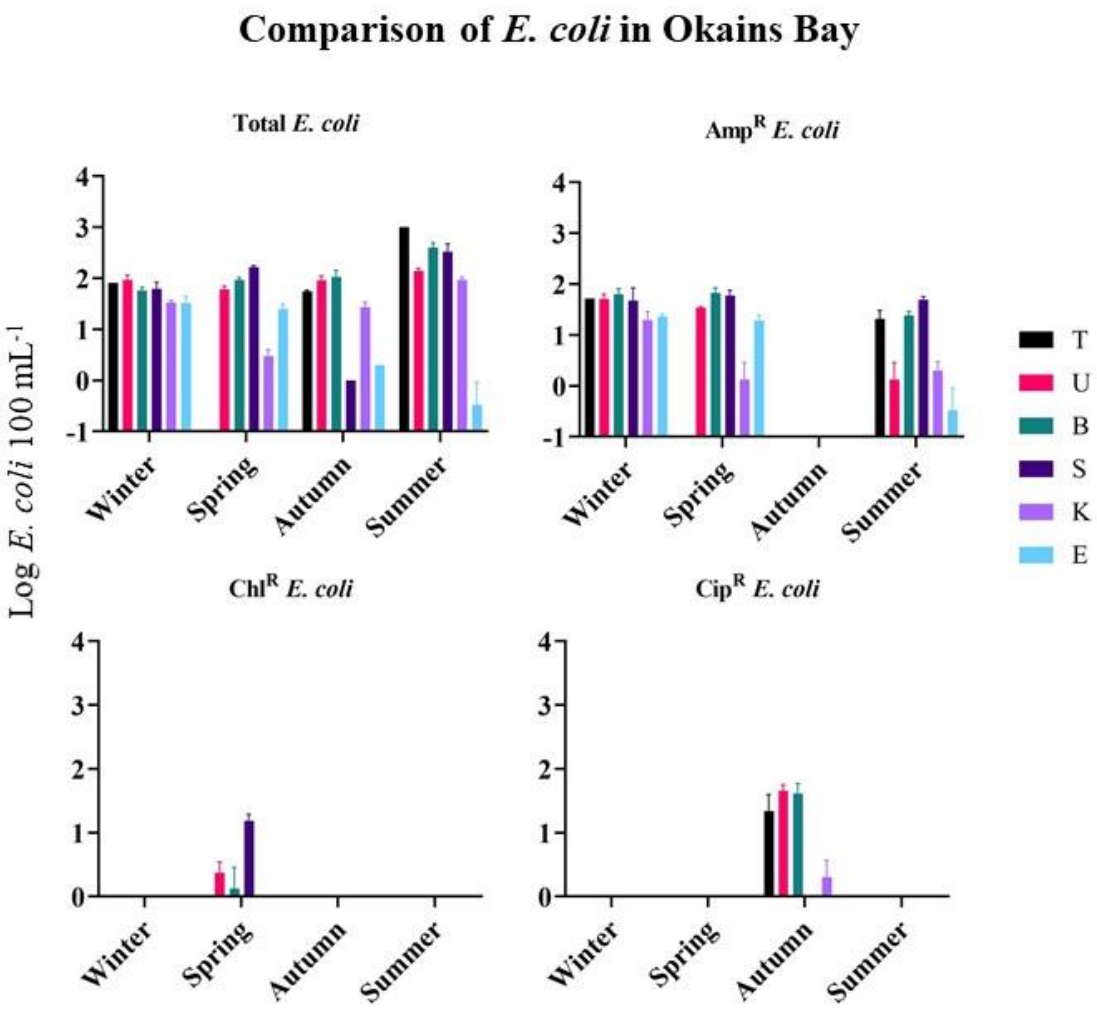


Figure 6 Log distribution of *E. coli* across four seasons in Okains Bay. Amp^R = ampicillin resistant; Chl^R = chloramphenicol resistant; Cip^R = ciprofloxacin resistant; T = Tributary; U = Upstream; B = bridge; S = Schoolhouse; K = Kitchen; E = Estuary.

2.15.2 Difference across sites

Overall, during the winter season there was a downward trend in *E. coli* counts going from upstream to downstream (site E) with the most upstream site (U) harbouring significantly more *E. coli* than the most downstream site (E; $p < 0.0001$). Ampicillin resistant *E. coli* followed the same trend with the exception of site T which had the lowest ampicillin resistant counts across the sites. The most upstream site had significantly more *E. coli* than the most downstream site ($p < 0.0001$). Chloramphenicol and ciprofloxacin resistant *E. coli* were below the detection limit in winter. The statistical output from the GLM with differences between each site can be found in Appendix A8.

During the spring season, site T was too low to sample from. This site was omitted from the analysis. All sites had significantly different *E. coli* counts from each other ($p < 0.0001$). For both *E. coli* and ampicillin resistant *E. coli*, concentrations appear to increase from the most upstream site to the schoolhouse (S) before dropping significantly at site K and increasing again at site E (Figure 6). Chloramphenicol resistant *E. coli* was detected at sites U, B and S, with *E. coli* counts increasing going from upstream to downstream. Ciprofloxacin resistant *E. coli* were below the detection limit in spring. The statistical output from the GLM with differences between each site can be found in Appendix A9.

During the autumn season, the schoolhouse had relocated to a new building and the sample obtained from the old schoolhouse was inadequate, so this site was omitted from the analysis. All sites had significantly different *E. coli* counts from each other ($p < 0.0001$), apart from sites B and U. Like the spring season, *E. coli* and ciprofloxacin resistant *E. coli* concentrations increased going downstream until decreasing significantly at site K. Chloramphenicol and ampicillin resistant *E. coli* were below the detection limit in autumn. The statistical output from the GLM with differences between each site can be found in Appendix A10.

During the summer season, the *E. coli* concentrations generally decreased from upstream to downstream. All sites had significantly different *E. coli* counts from each other ($p < 0.0001$). Ampicillin resistant *E. coli* concentrations significantly increased going downstream until site S, before decreasing towards site E. Chloramphenicol and ciprofloxacin resistant *E. coli* were

below the detection limit in summer. The statistical output from the GLM with differences between each site can be found in Appendix A11.

2.16 Can *E. coli* persist on toothbrushes?

There were no significant differences between the methods of water exposure. There was no significant difference between the toothpaste and no toothpaste conditions. *E. coli* was only detected on the toothbrushes when they were embedded in agar immediately after exposure to the water and was only detected in the full immersion and aerosol methods. The *E. coli* counts detected were always below 10 cfu 20 mL^{-1}. In the consistent flow method, *E. coli* was below the detection limit at all time points (3, 6 and 18 h). Figure 7 shows the *E. coli* growth from when the toothbrushes were embedded in agar immediately after exposure to the water.

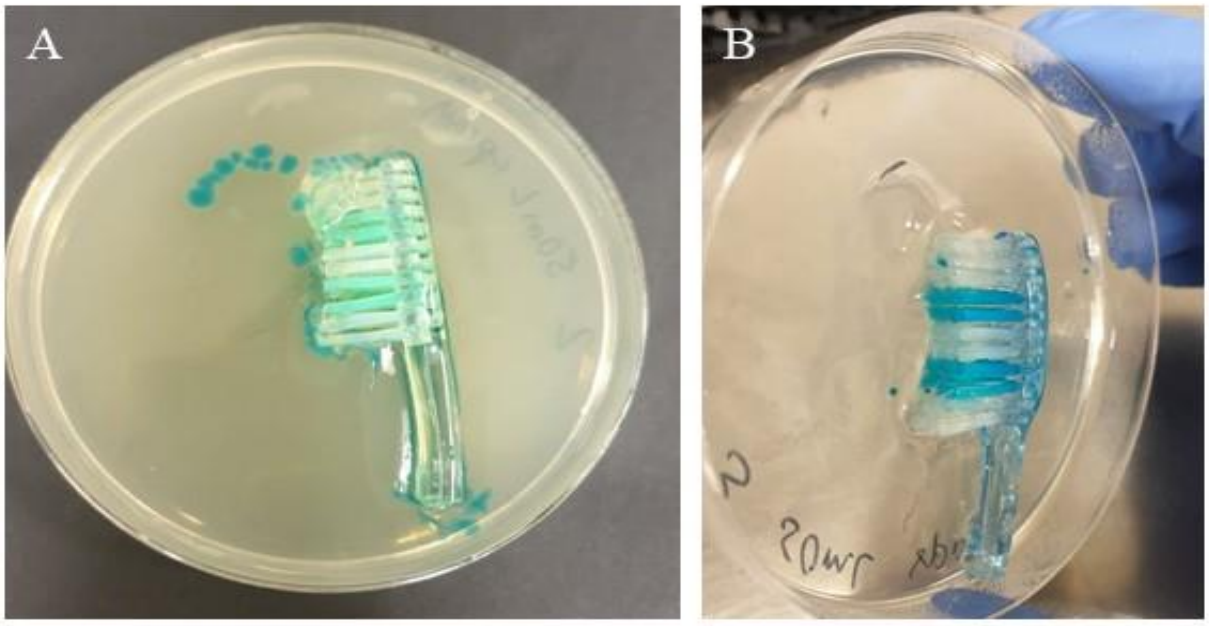


Figure 7 Toothbrush heads which were immediately embedded in TBX agar after exposure to Okains Bay campground water through the aerosol method. *E. coli* colonies are blue. A) Shows dispersal of *E. coli* like associated with excess water on the toothbrush head. B) *E. coli* colony embedded within the toothbrush bristles and around the toothbrush head.

Discussion

The airplane water storage tank analysis revealed that water from airplanes on long haul flight routes had significantly higher mesophilic bacterial concentrations than planes on domestic routes. *E. coli* concentrations were always below the detection limit. The limiting factor of this study was obtaining water samples from airplanes. Domestic flight samples were predominantly from main cities, which limited the analysis to similar size planes and may not have captured the true scope of microbiological quality in domestic aircrafts within New Zealand. Samples from long haul international flights were difficult to obtain, and in 2020 it was no longer an option, which limited the total number of samples obtained from long haul flights to only four. However, the difference observed in my analysis supports findings from Handschuh et al. (2015) who also identified that the water in planes used for long haul flights had poorer microbiological quality than short haul flights. The results from this analysis do not support my first hypothesis, as no *E. coli* were detected in either long haul or domestic flights. Without identifying the isolated bacteria, it is difficult to accurately compare the risk presented by the water from either long haul or domestic flights. This would be the next step if this analysis were done in the future. Techniques such as metabarcoding may be a good option for this.

Overall, the results from the water quality analysis showed that Treated had significantly lower *E. coli* concentrations and ampicillin resistant *E. coli* concentrations than Stream. *E. coli* concentrations in water from the three regions were not significantly different, however, there were significant differences within Canterbury. Mesophilic bacterial counts were significantly lower in Treated and had the highest counts in Banks Peninsula and the lowest in Marlborough (Table 6).

The significant differences in *E. coli* and ampicillin resistant *E. coli* between Treated and Stream supports other findings that robust water treatment systems need to be in place to ensure that potable water is available (Phiri et al., 2021). This result partially supports my second hypothesis. For my hypothesis to be fully supported, I would have expected Other to harbour significantly more *E. coli* than Treated. The category Other included sources such as tributaries, rivers and springs, all of which had varying concentrations of *E. coli*. This category was created

because those sources were not sampled as frequently as Treated or Stream. Sources such as springs may not be subjected to the same anthropogenic impacts as rivers or tributaries, so the representation in this category may be skewed. There were no significant differences in *E. coli* counts between the three regions. The sites in the Banks Peninsula region were located in heavy agricultural areas with farming in the catchment area. Some of the sites were in small rural communities which were geographically distant from main townships and were likely to have a poor infrastructure for their drinking water and waste treatment. Water quality in this region has been previously identified as not meeting DWSNZ standards. The sites in the Canterbury region were all relatively close to a township (either Kaikoura or Christchurch), except for the Sharplin Falls car park site. This site had the highest *E. coli* concentration across all the sites in Canterbury. This site is in close proximity to Mt Somers station, which is a 3800 ha farm which farms sheep, deer and dairy and beef cows. In the Marlborough Sounds, the sites were all along the East coast of the Marlborough region, and were in close proximity to a township (either Blenheim or Picton). These sites are in a mixture of agriculture and forestry-based use areas, however, the Canterbury area (including Banks Peninsula) has more than four times the land use for agriculture (Ministry for the Environment, 2018). The sites Whites Bay and Whatamango Bay have previously been a part of a water quality study which also found that the *E. coli* concentrations in the drinking water were non-compliant with DWSNZ (Phiri et al., 2021). My findings were similar, with both sites consistently non-compliant with DWSNZ over multiple sampling events. The lack of significant differences between the water sources between regions may indicate that each region is subjected to its own influences of *E. coli* and resistant *E. coli* contamination.

Ampicillin resistant *E. coli* were the most consistent resistant populations detected across the sites and were detected at a much higher frequency than chloramphenicol and ciprofloxacin resistant *E.* coli. This aligns with Van Hamelsveld et al. (2019) and Adewale (2018) who found also found ampicillin resistant populations at a higher frequency than chloramphenicol and ciprofloxacin resistant populations in rural surface waters. The consistent detection of ampicillin resistant *E. coli* populations in this study was not surprising. Penicillin is one of the largest classes of antibiotic used in agriculture and veterinary medicine, with predominant use in cattle followed by companion animals (New Zealand Food Safety, 2020), and it is the most common

antibiotic used in dairy and meat production (Bryan & Hea, 2017). Ampicillin resistant *E. coli* were detected at sites that were in areas with heavy agricultural influences. I wanted to observe whether total *E. coli* could be predictive of resistant populations in the environment. Ampicillin resistant populations were selected for this analysis as it was the most consistent resistant population detected. This relationship has relevance for water quality monitoring as currently, antibiotic resistant populations are not monitored in the environment. If *E. coli* concentrations were predictive of resistant populations, it provides another dimension to water quality monitoring, where resistance information could potentially be extrapolated from total *E. coli* counts. Unfortunately, total *E. coli* concentrations was not predictive of ampicillin resistant populations. This means that total *E. coli* could not be used as a predictor of resistance in the environment. Ampicillin resistant *E. coli* were significantly different between Treated and Stream. In most cases, ampicillin resistant populations were identified at sites where the drinking water was sourced from Stream or Other and in agricultural areas.

Chloramphenicol resistant populations were only identified in one sampling event; however, this detection was not significant. In New Zealand, the use of chloramphenicol to treat bacterial infections in food producing animals is not permitted (New Zealand Food Safety, 2018). It is predominantly used to treat bacterial conjunctivitis in humans and in companion animals (Health Navigator, 2017; New Zealand Veterinary Association, 2018b; Ponen, 2019a). Though the use of chloramphenicol in New Zealand is limited to human and veterinary medicine, resistant populations still exist in the environment even if my analyses detected them infrequently. Chloramphenicol resistance in aquatic environments could be due to genes conferring resistance being shared with other bacteria in the environment, co-selection or cross-resistance or due to multidrug efflux pumps in bacterial systems (Yoo et al., 2003).

Interestingly, ciprofloxacin resistant populations were only identified during one sampling period in the Banks Peninsula region (May 2020). The most significant event which occurred at the time was the nation-wide lockdown due to the COVID-19 pandemic. Ciprofloxacin is secreted through urine, with 50-70% excreted unchanged. This may be a source for chemical selection of ciprofloxacin resistant *E. coli* as a result of the month-long lockdown posing a heavier burden on septic tanks in the rural community. However, without having samples from right before the

lockdown period, it is difficult to infer that this was the cause. In 2014, ciprofloxacin was one of the top ten most consumed antibiotics in human healthcare in New Zealand (Williamson et al., 2016). It is also used to treat infections in companion animals and in agriculture (Ministry for Primary Industries, 2005). Its use is widespread in New Zealand.

In Okains Bay, there were significant seasonal effects on mesophilic bacteria, *E. coli* and ampicillin resistant *E. coli*. Autumn had significantly fewer *E. coli* and ampicillin resistant *E. coli* than winter and summer ($p < 0.0001$). Winkworth-Lawrence and Lange (2016) had previously detected correlations between high intensity farming in winter and antibiotic resistant bacteria. This reason may account for the high *E. coli* and ampicillin resistant *E. coli* during the winter season. The highest *E. coli* counts were detected in summer. The warmer conditions during summer have been found to contribute to persistence of *E. coli* (Oliver & Page, 2016). Along with this, Okains Bay is a popular location for visitors during summer. The higher *E. coli* concentrations may be due to a combination of both factors.

High concentrations of *E. coli* were consistently detected in the tributary (site T). This highlights one pathway of *E. coli* contamination into the Opara Stream. Through ESR, I was able to obtain a sample from one of the sources of the Opara Stream, a spring on Thacker's Farm. *E. coli* was detected in this source. This indicates contamination of the spring itself, however, as this was only a one-off sampling event, the results may not be truly representative. Frequent monitoring is needed to determine whether the presence of *E. coli* is consistent. Across all seasons, *E. coli* was detected at the most upstream site. This site is 5.3 km from the most downstream site. The increasing concentrations of *E. coli* and ampicillin resistant *E. coli* going downstream during some seasons indicates that there may be multiple input sources of *E. coli*. In future, gathering samples from further upstream than site U could provide a better insight as to potentially where contamination begins.

The old schoolhouse's UV irradiation system and filter seemed to fail consistently, leading to noncompliance for drinking water. *E. coli* concentrations were just above the detection limit at the schoolhouse in autumn (1 cfu 100 mL^{-1}). At this point the school had moved into a new building and had not used that building in months. In autumn, the other sites still had *E. coli*

concentrations well above the detection limit, and during the summer season, *E. coli* concentrations at the old schoolhouse were above the detection limit again, suggesting that the autumn observation was an artefact and was likely due to the water being stagnant in the pipes for some time.

The results from the toothbrush experiment showed that were no significant differences between methods of water exposure, and no significant differences between toothpaste and no toothpaste. *E. coli* could only be detected when enriched in LB agar immediately after exposure to the water. These results are not representative of the microbial load that may exist on toothbrush heads. The toothbrushes were sterile, which is not the case for regularly used toothbrushes. As observed with the embedded toothbrush heads, *E. coli* could be detected immediately after water exposure. Further analysis could determine the abundance of *E. coli* immediately after exposure to water through the sonication, vortex, and filtration steps to gather more quantitative information.

Conclusion

The results of this study showed that raw water that is influenced by different anthropogenic activities can harbour antibiotic resistant populations of *E. coli*. This was observed by the significant differences in Treated and Stream. As many sites were only sampled once, it is not possible to generalize that different land uses were the explanation for the differences between sites. However, treated drinking water harboured significantly lower *E. coli* than drinking water sourced from untreated streams in all regions, concluding that treatment of raw water in rural environments is essential for access to potable water. In the Okains Bay case study, seasons had a significant effect on *E. coli* concentrations across the sites, as well as resistant populations.

Chapter 3: Study on the prevalence and diversity of multidrug resistant *E. coli* in rural water sources

As discussed in Chapter 1 and 2, antibiotic resistance occurs at easily detectable frequencies in the New Zealand environment. This chapter focuses on the antibiotic susceptibility profiles of the *E. coli* isolates that were detected in the water quality experiments in Chapter 2 against eight antibiotics from seven different classes, as well as extended spectrum β-lactamase (ESBL) production, and *amp*C β-lactamase (AmpC) production. The ability of the *E. coli* isolates to share genetic material conferring antibiotic resistance is also explored. The data explored in this chapter contributes to the growing body of evidence that MDR bacteria are present in our environment.

3.1 Antibiotic classifications

Antibiotics from eight antibiotics from seven different classes were used for this work. All eight antibiotics are on the WHO's Model List of Essential Medicines (World Health Organisation, 2019b), and are classed as 'highly' or 'critically' important for human therapeutics (World Health Organisation, 2018). The WHO released the '2019 AWaRe Classification Antibiotics database, where it classifies 180 antibiotics and antibiotic combinations into the following groups: 'Access', 'Watch', and 'Reserve' (World Health Organisation, 2019a). A description of each group is shown in Table 7.

Table 7 Description of the WHO's 2019 AWaRE Classification Antibiotics groups. All of the information in this table was obtained from WHO, 2019c.

Group	Description
Access	Antibiotics that have activity against a broad range of susceptible pathogens while also showing lower resistance potential than antibiotics in other groups.
Watch	Antibiotics that have higher resistance potential and/or have a relatively high risk of selection for bacterial resistance.
Reserve	Antibiotics that should be reserved for treatment of confirmed or suspected infections. These should be treated as 'last resort' options used for specific situations where alternatives have failed.

3.2 Introduction to antibiotics used in this thesis

Antibiotics are often used in both human healthcare and veterinary medicine. This makes it important to monitor antibiotic resistance in both clinical and environmental isolates as resistant zoonotic pathogens can cause serious problems for treatment of common infections. Figure 8 shows the antibiotics used in my thesis research and how they are used in New Zealand. Half of the eight antibiotics are used in humans, companion animals and livestock, and all four antibiotics used on livestock are used on both humans and companion animals.

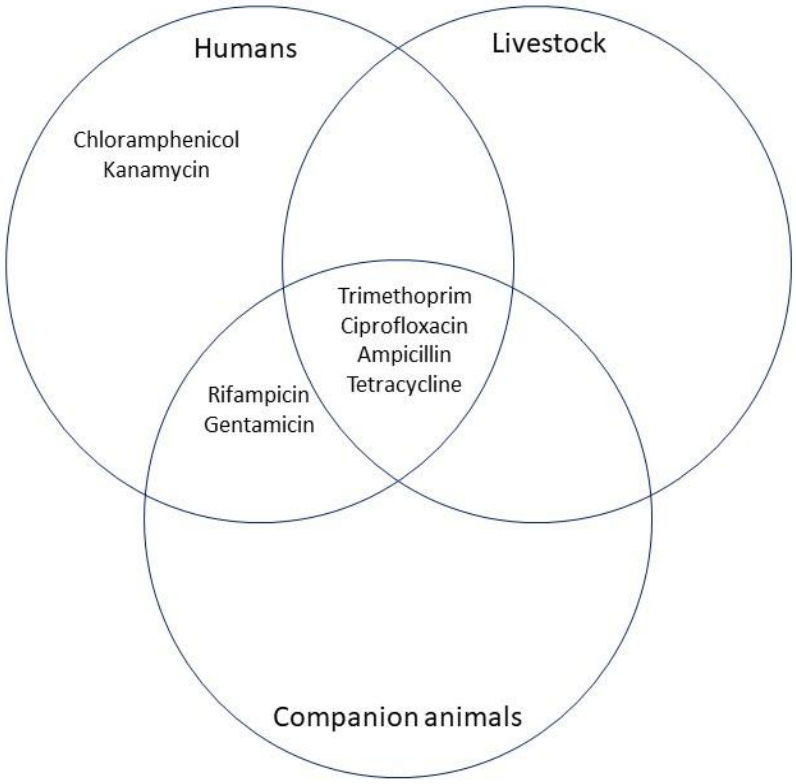


Figure 8 The antibiotics used in this thesis research and the different sectors in which they are used in in New Zealand.

3.2.1 Rifampicin

Rifampicin is an antimycobacterial antibiotic but is also effective against a large range of bacterial pathogens. It remains an important alternative antibiotic for infections caused by pathogens such as MRSA (Moellering, 2008). It is classed as a ‘Critically Important

Antimicrobial' and has been placed in the 'Watch' group (World Health Organisation, 2018, 2019b). Rifampicin inhibits RNA polymerase, thereby preventing transcription (Wehrli, 1983). Rifampicin resistance is generally caused by mutations in the rifampicin resistance determining region (RRDR), a 81bp region in the *rpoB* gene (Goldstein, 2014). In New Zealand, rifampicin is used to treat bacterial infections in humans and animals (Ministry for Primary Industries, 2005; Ministry of Health, 2019b; Page, 2017). Rifampicin is excreted through urine and faeces with less than 30% excreted unchanged (Wishart et al., 2018). New Zealand has a low incidence of clinically relevant rifampicin resistance. In 2004, 1% of *Mycobacterium tuberculosis* and *M. bovis* isolated from TB positive patients were resistant to rifampicin (Heffernan, 2004), and between 2007 and 2016, no significant trends in resistance to rifampicin have been observed (Institute of Environmental Science and Research, 2019). In 2010, the first case of extensively drug resistant (XDR) TB was identified in New Zealand (Goh et al., 2011). No other reports of XDR TB in New Zealand have been identified since.

3.2.2 Trimethoprim

Trimethoprim is an antifolate antibiotic. It is classed as a 'Highly Important Antimicrobial' and has been placed in the 'Access' group (World Health Organisation, 2018, 2019b). Trimethoprim binds to dihydrofolate reductase (DHFR), preventing the reduction of dihydrofolic acid (DHF) to tetrahydrofolic acid (THF), inhibiting the synthesis of folic acid and DNA (Brogden et al., 1982). In New Zealand, trimethoprim is used to treat bacterial infections in humans, companion animals, and livestock (Ministry for Primary Industries, 2005; New Zealand Veterinary Association, 2018a). It is commonly used in conjunction with sulphonamides. In 2014, it was one of the top ten most heavily consumed antibiotics in human healthcare in New Zealand (Williamson et al., 2016). Trimethoprim can be purchased without prescription from registered pharmacists (Gauld et al., 2017; Ponen, 2019b). Trimethoprim is excreted unchanged through urine, with approximately 50-60% of trimethoprim excreted within 24 hours of oral administration (Health Canada Product Monograph, 2014).

Trimethoprim resistance is increasing in New Zealand. Between 2007 and 2015, there was an increase in trimethoprim resistance in clinical isolates, though this increase was not associated with the change in trimethoprim availability that stemmed from changes in prescription

regulation in 2012 (New Zealand Medicines and Medical Devices Safety Authority, 2018). In 2017, 25.9% of non-ESBL-producing *E. coli* and 69.8% of ESBL-producing UTI *E. coli* isolates from humans in New Zealand were resistant to trimethoprim (Institute of Environmental Science and Research, 2017a). These data support the relationship between trimethoprim resistance and ESBL-producing bacteria that have been seen in clinical isolates in other studies (Auer et al., 2010; Critchley et al., 2019; Rodríguez-Baño et al., 2018). In comparison, trimethoprim resistant *E. coli* isolated from UTIs from companion animals decreased from 6.5% in 2005 to 2.8% in 2012 (McMeekin et al., 2017). In dairy cows, 0.5-17% of *Staphylococcus* spp. were resistant to trimethoprim/sulfamethoxazole (McDougall et al., 2014).

3.3.3 Gentamicin and kanamycin

Gentamicin and kanamycin are aminoglycoside antibiotics, and both are classed as 'Critically Important Antimicrobials'. Gentamicin has been placed in the 'Access' group and kanamycin has been placed in the 'Watch' group (World Health Organisation, 2018, 2019b). Aminoglycosides inhibit protein synthesis in bacteria, negatively impacting translation of mRNA (Davies & Davis, 1968). Aminoglycoside sales for agriculture and horticulture in New Zealand have decreased over the last few years. The distribution of aminoglycoside sales in 2017 was horticulture (69%), livestock species (pigs, sheep, cattle; 24%) and companion animals, including horses (7%) (New Zealand Food Safety, 2019). In New Zealand, gentamicin is used to treat bacterial infections in humans and companion animals (CDHB Antimicrobial Stewardship Committee, 2020; Ministry for Primary Industries, 2005; Page, 2017). while, kanamycin is used as a second line treatment for tuberculosis (Ministry of Health, 2019b). Gentamicin is excreted unchanged in urine, with 70% or more unchanged gentamicin recovered in urine within 24 hours of administration (Wishart et al., 2018). Similarly, kanamycin is excreted unchanged through urine (Kunin, 1966). Gentamicin resistance has been observed in clinical human bacterial isolates in New Zealand, and at low frequencies in the environment (Adewale, 2018; Institute of Environmental Science and Research, 2017a; Schousboe et al., 2015). In clinical isolates, gentamicin resistance is detected more frequently in ESBL-producing *Enterobacter* spp. than in non ESBL-producers (Institute of Environmental Science and Research, 2017a). No data was available regarding the detection of kanamycin resistance in New Zealand.

3.3.4 Chloramphenicol

Chloramphenicol is an amphenicol antibiotic and it is classed as a 'Highly Important Antimicrobial' and has been placed in the 'Access' group (World Health Organisation, 2018, 2019b). Chloramphenicol binds to the 50S ribosomal subunit in bacteria, suppressing peptidyl transferase activity and interfering with protein synthesis (Wishart et al., 2018). In New Zealand, chloramphenicol is used to treat bacterial infections in humans. Use in animals is not permitted (New Zealand Food Safety, 2018). It is the first-choice treatment for bacterial conjunctivitis and can be obtained without a prescription from a registered pharmacist (Health Navigator, 2017; Ponen, 2019a). Chloramphenicol is predominantly excreted through urine as the inactive metabolite chloramphenicol glucuronate, with between 5-15% excreted unchanged through urine (Ambrose, 1984). Chloramphenicol resistance is not common in New Zealand, but it has been observed before. Clinical isolates of chloramphenicol resistant *Streptococcus pneumoniae* were identified at a rate of 1.2 - 3.4% between 2001 and 2010 (Heffernan, 2011; Ikram, 2010). Amongst non-typhoidal *Salmonella*, chloramphenicol resistance was observed in 2.5% of human isolates but not in environmental isolates (Institute of Environmental Science and Research, 2016). In *Shigella* spp., chloramphenicol resistance was observed in 28.5% of clinical isolates tested between 2015 and 2016 (Institute of Environmental Science and Research, 2017b).

3.3.5 Ciprofloxacin

Ciprofloxacin is a fluoroquinolone antibiotic. It is classed as a 'Critically Important Antimicrobial' and has been placed in the 'Watch' group (World Health Organisation, 2018, 2019b). Ciprofloxacin inhibits DNA replication by targeting the essential type II topoisomerase enzymes DNA gyrase and topoisomerase IV and can also inhibit DNA transcription and translation at some concentrations (Jacoby, 2005). The emergence of resistant populations is predicted to be a result of the extensive use of ciprofloxacin, as well as other fluoroquinolones (Hooper, 2001). This is due, in part, to chromosomal mutations in the target enzymes DNA gyrase and topoisomerase IV, as well as mutations which alter the expression of diffusion channels and efflux systems (Hooper & Jacoby, 2016). In New Zealand, ciprofloxacin is used to treat bacterial infections in humans, animals, and in livestock (Ministry for Primary Industries, 2005; New Zealand Medicines and Medical Devices Safety Authority, 2012). In 2014, it was one

of the top ten most heavily consumed antibiotics in human healthcare in New Zealand (Williamson, 2016). Ciprofloxacin is excreted unchanged through urine and faeces, with higher concentrations detected in faeces than in urine (LeBel, 1988). Trends of ciprofloxacin resistance varies across clinical isolates in New Zealand, however, overall, it is slowly increasing (Heffernan et al., 2018; Wellington SCL, 2020). Ciprofloxacin resistance is also strongly associated with ESBL-production. This has been observed globally, as well as in New Zealand (Institute of Environmental Science and Research, 2017a).

3.3.6 Ampicillin

Ampicillin belongs to the antibiotic class penicillin. It is classed as a 'Critically Important Antimicrobial' and has been placed in the 'Access' group (World Health Organisation, 2018, 2019b). Penicillins were amongst the most purchased classes of antibiotic in New Zealand between 2011 and 2017, and they had the highest use in companion animals and in cattle (New Zealand Food Safety, 2020). Penicillin prevents cell wall synthesis by binding to penicillin-binding proteins (PBPs), inhibiting cell wall peptidoglycan synthesis (Yocum et al., 1980). In New Zealand, ampicillin is used to treat bacterial infections in humans, companion animals, and in livestock (Baoumgren, 2019; Bryan & Hea, 2017; Ministry for Primary Industries, 2005; The New Zealand Veterinary Association, 2018). Ampicillin is excreted unchanged through urine, bile, and faeces (Wishart et al., 2018). Ampicillin resistance has been observed in livestock (Nulsen et al., 2008), and in the New Zealand environment (Adewale, 2018; Schousboe et al., 2015; Van Hamelsveld et al., 2019).

3.3.7 Tetracycline

Tetracycline belongs to the tetracycline family of antibiotics. It is classed as a 'Highly Important Antimicrobial' and has been placed in the 'Access' group (World Health Organisation, 2018, 2019b). Tetracycline inhibits protein synthesis by binding to the 30S ribosomal subunit (Chopra & Roberts, 2001). In New Zealand, tetracycline is used to treat a wide range of infections in humans, companion animals and livestock (Best Practice Advocacy Centre New Zealand, 2012; New Zealand Food Safety, 2020; New Zealand Veterinary Association, 2018b). Tetracycline is excreted unchanged in high concentrations through urine and faeces (Wishart et al., 2018).

Tetracycline resistance has been observed in New Zealand in surface waters (Adewale, 2018; Van Hamelsveld et al., 2019), in livestock (French et al., 2019; Nulsen et al., 2008), and in human clinical isolates (Institute of Environmental Science and Research, 2017a).

3.3 Extended Spectrum β-Lactamases (ESBL) and AmpC β-Lactamases (AmpC)

Extended spectrum β-lactamase-producing (ESBL) bacteria and AmpC β-lactamase-producing (AmpC) bacteria are important threats to how antibiotics are used to treat infections, and are often associated with poor treatment outcomes, higher morbidity, and mortality, and increase health care costs (Brook, 2009; Drinkovic et al., 2015; Jacoby, 2009). β-lactamases are hydrolytic enzymes that confer resistance to β-lactam antibiotics such as penicillins and cephalosporins by cleaving the beta-lactam ring (Dhillon & Clark, 2012). They can be chromosomal or plasmid genes and can confer resistance to a wide array of commonly used non β-lactam antibiotics (Brook, 2009; Toombs-Ruane et al., 2017). ESBL-producers are inhibited by β-lactamase inhibitor (BLI) combinations while AmpC βL-producers are resistant to BLI combinations but are usually sensitive to carbapenems (Grover et al., 2013; Jacoby & Han, 1996).

ESBL and AmpC βL enzymes are primarily produced by members of the *Enterobacteriaceae* family and display stronger resistance phenotypes than non-producers (Grover et al., 2013; Jacoby, 2009). This has been seen in clinical isolates in New Zealand, as discussed above. ESBL-producing *E. coli* can pose a significantly higher risk of complications to humans than non-ESBL-producing *E. coli* (Briñas et al., 2005; Melzer & Petersen, 2007). Ampicillin, ciprofloxacin, gentamicin, and trimethoprim resistance have been previously associated with ESBL-producing bacteria (Grover et al., 2013; Liu et al., 2011; Paterson & Bonomo, 2005). Ampicillin is a β-lactam antibiotic that is susceptible to β-lactamase hydrolysis, however, it is suggested that genes conferring resistance to ciprofloxacin and gentamicin may share a conjugative plasmid with genes encoding for ESBLs (Grover et al., 2013).

In New Zealand, ESBL- and AmpC βL-producing bacteria have been found in hospitals, in the community and in the environment. They have been identified in humans, companion animals and in livestock. Between 2005 and 2014, ESR conducted one-month long surveys of ESBL-producing *Enterobacteriaceae* from hospital and community patients. Their latest report found that between 2007 and 2016, ESBL-producing *E. coli* populations increased from 52% to 74.1% (Heffernan, 2018). Further, the ESBL-producing *E. coli* populations were predominantly found in community patients (68.1%), with more than half of the populations resistant to ciprofloxacin (62.6%) and trimethoprim (66.7%). In the Auckland community, the prevalence of AmpC-producing *E. coli* has increased in recent years. In 2011, genetically unrelated AmpC-producing *E. coli* were identified in human urine samples, and they were significantly more resistant to other antibiotics than their non-AmpC βL-producing counterparts (Drinkovic et al., 2015). ESBL- and AmpC-producing bacteria have been found in clinical isolates from companion animals in New Zealand as well. Karkaba et al. (2017) identified ESBL- and AmpC-producing *Enterobacteriaceae* that were often MDR in companion animals and found that these isolates were resistant to more antibiotics than non ESBL- and non AmpC βL-producing isolates. In livestock, the prevalence of ESBL- and AmpC βL-producing *E. coli* is low, however, they are still detected (Burgess et al., 2021). In New Zealand waterways, Adewale (2018) and Van Hamelsveld et al. (2019) identified ESBL-producing *E. coli* in the Ōtākaro/Avon river, an urban river which runs through Christchurch, and Silverstream, a rural stream that drains an area used for agriculture. Their results demonstrate that ESBL-producing *E. coli* are present in both urban and agricultural environments.

3.4 Spreading of antibiotic resistant genes

Horizontal gene transfer has been recognised as a contributor to the declining efficiency of antibiotics (D'Costa et al., 2006). Conjugation is one of the principle routes that aids the dissemination of antibiotic resistance genes, as conjugative plasmids often harbour multiple antibiotic resistance genes (Wozniak & Waldor, 2010), and in *Enterobacteriaceae*, conjugation is the most common mechanism for spreading antibiotic resistance genes (Bethke et al., 2020). The spread of antibiotic resistance in hospitals and communities have largely been the focus of antibiotic stewardship, however the natural environment is also a reservoir of resistant determinants and resistant bacteria (Abe et al., 2020). A proportion of antibiotics that are

consumed in humans, animals and livestock can often be excreted unchanged into the environment. These residues can end up in our waterways, contributing to the antibiotic 'resistome' (Figure 1). Antibiotic resistance and resistant determinants can be detected in environments without selective pressure, suggesting that they persist in those environments through bacterial populations (Abe et al., 2020; Gullberg et al., 2011). Aquatic environments represent large reservoirs of antibiotic resistant genes and include a mixture of clinical, terrestrial, and human commensal bacteria (Abe et al., 2020). Some commensal bacteria are opportunistic pathogens, which becomes an issue when they harbour antibiotic resistant genes that they may have acquired from the environment (D'Costa et al., 2006). The human gastrointestinal tract is a prime location for horizontal gene transfer, which includes transfer of antibiotic resistance genes to other pathogens (Broaders et al., 2013). When relating this back to our tourist visiting rural New Zealand, being exposed to MDR bacteria from surface waters has more serious implications as the environmental bacteria and gut bacteria may interact, further disseminating resistant determinants or alternatively, they may pick up a MDR pathogen leading to difficulties when it comes to treatment.

3.5 Main hypothesis

Hypothesis 1: Tourists will be exposed to more MDR *E. coli* than recognised because of their concentrated experience with water supplies that are rarely or never monitored. This was tested by measuring the concentration of both *E. coli* and MDR *E. coli* in these water sources.

Hypothesis 2: MDR *E. coli* isolated from the environment will be able to share resistant determinants with susceptible bacteria through horizontal gene transfer. This hypothesis will be supported if resistance is observed in a susceptible lab strain after conjugation experiments. This hypothesis would not be supported if sharing of resistance genes is not observed.

Hypothesis 1 will be tested by comparing the antibiotic susceptibility profiles of *E. coli* isolates from the three drinking water sources: Treated, Stream and Other. Hypothesis 2 will be tested by qualitative conjugation experiments.

Methods

3.6 Antibiotic Susceptibility Assay

E. coli isolates from the water quality experiments in Chapter 2 and a standard laboratory strain and susceptible environmental strain (Table 8) were used for the antibiotic susceptibility assay. Antibiotics were purchased from Becton Dickinson, Sigma, Oxoid or Duchefa, and liquid stock solutions were prepared according to the manufacturers' instructions. Random representative populations were selected from TBX only, TBX supplemented with ampicillin, TBX supplemented with ciprofloxacin, and TBX supplemented with chloramphenicol plates. The antibiotic susceptibility assay is based on Adewale (2018) and Van Hamelsveld et al. (2019) with minor modifications. Liquid LB (10 ml) was inoculated with the isolates and grown with aeration to saturation in a 37℃-shaking incubator. 4 µL aliquots were transferred to LB plates supplemented with antibiotics. The concentrations used and the clinical breakpoints as per the Clinical and Laboratory Standards Institute (CLSI) guidelines are shown in Table 9 (Clinical and Laboratory Standards Institute, 2020). Isolates were tested up to their minimum inhibitory concentrations (MIC).

Table 8 Bacterial strains used as controls in this study.

Strain	***Species***	**Genotype**
CMB41	*E. coli*	Environmental strain. Susceptible to all antibiotics in used in antibiotic susceptibility assay
JB570	*E. coli*	Lab strain. *hsdS*, *leuB6*, *thr*, rifampicin resistance

Table 9 Antibiotics used and their concentration range to determine resistance.

Antibiotic	**Concentration Range Tested** μg mL^{-1}		
	Susceptible	Intermediate	Resistant
Ampicillin	≤ 8	16	32 ≤
Kanamycin	≤16	32	64 ≤
Gentamicin	≤ 4	8	16 ≤
Tetracycline	≤ 4	8	16 ≤
Chloramphenicol	≤ 8	16	32 ≤
Ciprofloxacin	≤ 0.25	0.5	1 ≤
Rifampicin	-	-	100
Trimethoprim	≤ 8	-	16 ≤

3.7 Extended Spectrum β-Lactamase (ESBL) and AmpC β-Lactamase (AmpC β-L) Screening

104 *E. coli* isolates that were resistant to ampicillin (≥16 μg/mL) and/or ciprofloxacin (≥1 μg/mL) were screened for ESBL production. Strains were transferred from frozen stocks by streaking onto plates of LB agar and incubated for 16 h in a 37°C incubator. The following day, a single colony was chosen and grown in 10 mL liquid LB for 4 hours at 37°C or until OD_{600} reached 0.5. 200 μL of culture was spread onto LB agar in a petrie dish and left to dry for five minutes. After this drying period, the following antibiotic discs were placed as shown in Figure 9: 1) cefotaxime 30 μg, 2) ceftazidime 30 μg, 3) cefotaxime and clavulanic acid 30/10 μg, and 4) ceftazidime and clavulanic acid 30/10 μg. The antibiotic susceptibility discs used were BD BBL™ Sensi-Discs™ purchased from Becton Dickinson. Resistance to cefotaxime and ceftazidime were determined as per the CLSI guidelines, and a positive result for ESBL production was indicated by a growth difference of ≥5 mm between cefotaxime and) cefotaxime and clavulanic acid, and ceftazidime and ceftazidime and clavulanic acid (Clinical and Laboratory Standards Institute 2020).

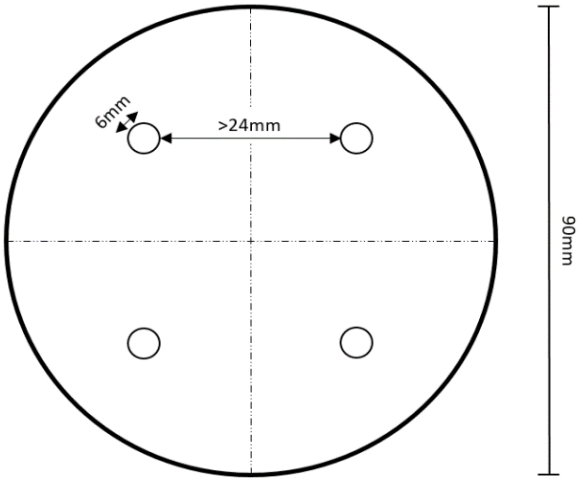


Figure 9 Placement of antibiotic discs in ESBL and AmpC screening assay.

3.8 Conjugation experiments

Antibiotic resistance gene transmission by conjugation was observed qualitatively and quantitatively. Isolates were screened for donor activity using a qualitative assay. Those that had a detectable donor activity were chosen for the quantitative assay.

3.8.1 Qualitative Conjugative Experiments

A procedure based on Van Hamelsveld et al. (2019) and Heinemann and Ankenbauer (1993) was used with modifications. Donor and recipient strains were streaked onto LB agar plates from frozen stocks and incubated 18 h at 37°C. For each donor, three different well-separated colonies were selected for replicates. The following day, a single colony was chosen, and the donor and recipient cultures were grown in 10 mL liquid LB until OD_{600} = 1 was reached. 10 µL of donor and recipient cultures were mixed together as a single 20 µL on LB agar, allowed to dry. Plates were incubated for 3 h at 37°C before replica plating onto LB agar containing rifampicin (100 µg mL^{-1}) and the donor antibiotic marker, and onto LB agar containing rifampicin (100 µg mL^{-1}) only. Plates were incubated for 16 h at 37°C. Putative transconjugants were purified and confirmed by streaking onto fresh antibiotic containing LB agar and incubated for another 16 h at 37°C. Antibiotic concentrations were determined from the results of the antibiotic susceptibility assay.

Prior to beginning the quantitative conjugation experiments, a PhD student in my lab group and I performed a series of experiments to observe which parameters would be optimal for conjugation to occur between the environmental and lab *E. coli* strains. This preliminary analysis stemmed from issues with low conjugation efficiency during quantitative conjugation experiments. Different growth parameters were explored; 1) temperature, 30°C vs 37°C, 2) multiple exponential growth phases to dilute conjugation repressors, and 3) mating times, 3 h versus overnight. We concluded that growth to two exponential phases (OD_{600} = 0.5) and overnight mating was sufficient to increase conjugation efficiency.

3.8.2 Quantitative Conjugative Experiments

Transmission rates were quantified for a sub-set of the environmental MDR *E. coli* isolates. *E. coli* JB570 was selected as the recipient strain (Table 8). The initial steps of the procedure for this experiment were the same as the qualitative conjugation experiments. The mating mix was in a liquid culture instead of solid agar, and donor and recipient cultures were mixed in fresh liquid LB at a ratio of 1:10, to ensure recipient concentration was not a limiting factor. This mating mix was incubated for 18 h in a non-shaking 30°C incubator. A dilution series was used to quantify the rate of transmission. The donor replicates were transferred to LB agar containing the donor antibiotic marker, and the transconjugants were transferred to LB agar containing rifampicin (100 μg mL^{-1}) and the donor antibiotic marker. Plates were incubated for 16 h at 37°C, and the following day, the transmission rate was determined by the number of transconjugants divided by the number of limiting parents (donor).

3.9 Statistical analysis

Across the three regions, I was interested MDR *E. coli* was dependent on the water source, and whether there were differences across the regions. The same sites that were used in the water quality analysis in Chapter 2 were used for this analysis. To compare the MDR isolates, the total counts of MDR per site were used instead of the concentration relative to total *E. coli.* This was because total *E. coli* was not found to be predictive of resistant populations. A GLMM with a Poisson log link was selected for analysis of MDR isolates because the MDR counts followed a Poisson distribution, the variance was not normally distributed, and the water sources categories

were unbalanced. Over dispersion was accounted for by assigning each observation a unique value and including this as a random effect in the analysis.

In Okains Bay, I was interested in whether there were seasonal effects on MDR *E. coli*, and whether there were differences across the sites. For both analyses, a GLMM with a Poisson log link was selected for analysis of MDR isolates because the MDR counts followed a Poisson distribution, the variance was not normally distributed, and the water sources categories were unbalanced. Over dispersion was accounted for by assigning each observation a unique value and including this as a random effect in the analysis.

Results

3.10 MDR assay

Treated had significantly fewer MDR *E. coli* than Stream ($p < 0.0454$). Other in Banks Peninsula had significantly more MDR *E. coli* than Treated in Canterbury ($p = 0.039$). Within Canterbury, Stream had significantly more MDR *E. coli* than Treated ($p = 0.0274$). The detailed statistical output from this analysis can be found in Appendix A12.

3.11 Antibiotic Susceptibility Assay

The following results include *E. coli* isolates from sites that were not included in the statistical analyses. See Appendix B for a list of MDR *E. coli* isolates from this analysis. A total of 535 *E. coli* isolates were tested against eight antibiotics in the antibiotic susceptibility assay. Among the tested isolates, 55% were directly isolated on TBX with no antibiotics supplemented. The others were isolated on either ampicillin, chloramphenicol, or ciprofloxacin. *E. coli* strains JB570 and CMB41 were used as controls (Table 8).

Of the 535 isolates, 15% were MDR. Among the MDR isolates, 1% were isolated from Treated, 46% were isolated from Other, and 53% were isolated from Stream. The highest numbers of multidrug resistance arose from TBX without supplemented antibiotics, closely followed by *E. coli* that were isolated on TBX supplemented with ampicillin (Table 10). Of the isolates tested, the most common resistance observed was ampicillin resistance and gentamicin resistance, followed by ciprofloxacin resistance (Table 10). Ampicillin and gentamicin resistance were most commonly observed together, followed by ampicillin and ciprofloxacin resistance.

Table 10 The number of isolates resistant to antibiotics used in the antibiotic susceptibility assay and the media which they were isolated on.

Isolated on	Amp	Chl	Rif	Tet	Kan	Tmp	Gen	Cip	Total MDR
TBX	79	8	15	17	3	3	93	85	**36**
Amp	85	1	11	6	16	2	74	19	**30**
Chl	4	4	1	2	5	1	4	2	**3**
Cip	3	3	2	1	0	7	0	63	**12**
Total	**171**	**13**	**29**	**26**	**24**	**13**	**171**	**169**	

Table 11 shows the distribution of resistances observed across the water sources and across the three regions. Although MDR was rare in Treated, its frequency amongst the *E. coli* found there was high. *E. coli* isolated from Treated had the highest proportion of resistant isolates (n =4, MDR = 25%), followed by Other (n = 196, MDR = 19%) and Stream (n = 330, MDR = 13%). In Banks Peninsula, the most common resistance observed was ciprofloxacin resistance, followed by gentamicin resistance. In Canterbury, the most common resistance observed was ampicillin resistance, followed by gentamicin and ciprofloxacin resistance. In Marlborough, the most common resistance observed was ampicillin resistance, followed by gentamicin resistance.

Table 11 The number of isolates resistant to antibiotics based on the water source.

Source	Total isolates tested (n)	Amp	Chl	Rif	Tet	Kan	Tmp	Gen	Cip	MDR
Other	196	52%	5%	2%	6%	6%	9%	40%	34%	19%
Stream	330	20%	1%	6%	4%	4%	2%	26%	31%	13%
Treated	4	50%	0%	50%	0%	0%	25%	0%	25%	25%
Banks Peninsula	370	24%	1%	4%	2%	5%	3%	32%	36%	14%
Canterbury	48	83%	0%	17%	58%	0%	0%	58%	58%	15%
Marlborough	117	54%	7%	9%	9%	3%	0%	31%	14%	14%

3.12 Extended Spectrum β-Lactamase Screening

A total of 104 *E. coli* strains that were resistant to ampicillin or ciprofloxacin were screened against cefotaxime, ceftazidime, and in combination with clavulanic acid. 33 (17%) isolates were resistant to cefotaxime, and 14 (14%) isolates were resistant to ceftazidime. Of the isolates resistant to cefotaxime, 5 (15%) were found to have a zone of inhibition greater than 5 mm around the cefotaxime/clavulanic acid antibiotic disc, which was a phenotypic confirmation of ESBL-producing *E. coli*. The remaining 32 (31%) isolates were phenotypically confirmed to be AmpC-producers. Of the ESBL- and AmpC-producers, 11 (30%) were resistant to both ampicillin and ciprofloxacin. I tested the possibility that ampicillin and ciprofloxacin resistance could be predictive of ESBL- and AmpC-production. Ampicillin resistance was not predictive of ESBL- or AmpC-production. Ciprofloxacin resistance was not predictive of EBSL-production, but it was predictive for AmpC-production ($p < 0.027$). ESBL and AmpC production was not associated with MDR in the tested isolates. A list of isolates that displayed a ESBL- or AmpC-producing phenotype can be found in Appendix C.

3.13 Conjugation

3.13.1 Qualitative conjugation experiments

Conjugative activity was observed in 64% of the 123 *E.* coli strains tested. Ampicillin and gentamicin resistance were most commonly found to be linked on a conjugative genetic element, followed by ampicillin and ciprofloxacin. Of the donors, 52%, 32%, 14% and 2% were resistant to one, two, three or four antibiotics, respectively. Only one isolate transferred all resistant markers (four resistant markers) to the recipient (1%). 14% transferred three resistant markers, and 33% transferred two resistant markers. Table 12 shows the isolates which showed a donor phenotype, and the resistant markers that were transferred to the susceptible *E. coli* JB570.

Table 12 Isolates showing a donor phenotype and the resistances observed in transconjugants after the conjugation experiments. Continued on the next page.

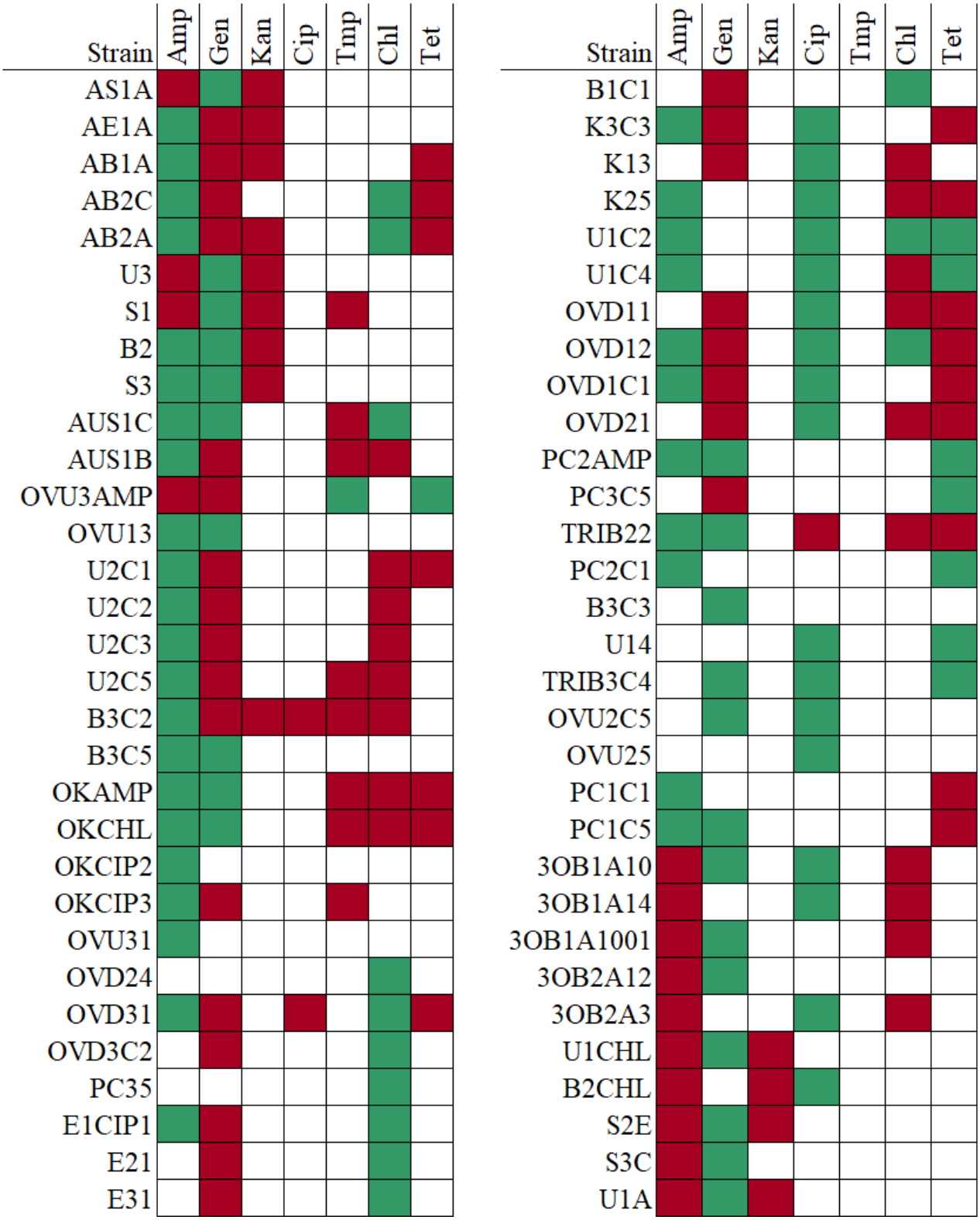


Table 2 continued.

Strain	Amp	Gen	Kan	Cip	Tmp	Chl	Tet
E2B							
AE2H							
1WHA13							
1WHA16							
1WHA18							
1WHA22							
WH24							
1WHA25							
1WHA29							
1WHA20							
OB17							
OB19							
OB21							
2OB22							
SF12							
SF13							
SF15							

Key

Transconjugant	
No Transfer Observed	
Donor Not Resistant	

3.13.2 Quantitative conjugation experiments

A total of 22 strains were tested in a quantitative conjugation assay. Donors sourced from drinking water taps were selected from amongst the donors tested in the qualitative conjugation assay. Conjugation frequencies ranged from 1 x 10^{-1} to 2.95 x 10^{-7} cfu mL^{-1} (Table 13). The highest frequency of conjugation observed was in *E. coli* strain 2OB22 which had an efficiency of plating (EoP) of 1 x 10^{-1} for gentamicin resistance. Overall gentamicin resistance was transferred at a higher frequency than ampicillin, chloramphenicol, and ciprofloxacin resistance. Cross resistance was observed in three strains.

Table 13 Results from the qualitative conjugation experiments. – indicates no observation of cross resistance.

Isolated from	***E. coli* strain**	**Resistant to**	**Transconjugant per limiting donor cfu mL^{-1} (EoP)**	**Cross resistance**
Okains Bay Residential Home	3OB1A10	Ciprofloxacin Gentamicin	1 x 10^{-5} 1 x 10^{-5}	Gentamicin Ciprofloxacin
	3OB1A1001	Gentamicin	3 x 10^{-4}	-
	3OB2A12	Gentamicin	2 x 10^{-4}	-
	OB21	Gentamicin Ampicillin	9 x 10^{-2} 1 x 10^{-3}	- -
	2OB22	Gentamicin	1 x 10^{-1}	-
Okains Bay School	S3CHL	Ampicillin Gentamicin	5 x 10^{-4} 5 x 10^{-4}	Gentamicin Ampicillin
	S1CHL	Gentamicin	2 x 10^{-4}	-
	AS1A	Gentamicin	8 x 10^{-3}	-
	AS2A	Ampicillin	3 x 10^{-2}	-
	S2E	Gentamicin	3 x 10^{-5}	-
Okains Bay Camp Kitchen	K3C3	Ampicillin Ciprofloxacin	5 x 10^{-5} 5 x 10^{-5}	Ciprofloxacin Ampicillin

Table 13 continued.

Sharplin Falls Carpark	SF12	Ampicillin	6×10^{-6}	-
		Gentamicin	4×10^{-5}	-
		Ciprofloxacin	3×10^{-5}	-
	SF13	Gentamicin	1×10^{-3}	-
		Ciprofloxacin	6×10^{-4}	-
	SF15	Gentamicin	6×10^{-5}	-
		Ciprofloxacin	2×10^{-5}	-
Whatamango Bay Campground	1WHA13	Ampicillin	3×10^{-7}	-
		Gentamicin	2×10^{-6}	-
		Chloramphenicol	2×10^{-7}	-
	1WHA16	Ampicillin	3×10^{-5}	-
		Gentamicin	2×10^{-4}	-
		Chloramphenicol	3×10^{-5}	-
	1WHA22	Gentamicin	2×10^{-4}	-
	1WHA24	Gentamicin	5×10^{-4}	-
		Ciprofloxacin	4×10^{-4}	-
	1WHA25	Gentamicin	5×10^{-5}	-
	1WHA29	Gentamicin	6×10^{-5}	-
	1WHA20	Gentamicin	5×10^{-5}	-
Whites Bay Campground	W3A6	Ciprofloxacin	7×10^{-4}	-

3.14 Case study: Okains Bay

In Okains Bay, I wanted to test whether seasons had a significant effect on MDR *E. coli* populations. I also wanted to test whether MDR *E. coli* varied by sampling site. The sites included in this analysis were tributary (T), upstream (U), bridge (B), school (S), campground kitchen (K), and estuary (E), which are sites along the Opara Stream, and residential home (RH)

which sources its drinking water from a tributary that feeds into the Opara Stream near the estuary.

Seasons had no effect on MDR *E. coli* observations along the Opara stream. Table 14 shows the antibiotic resistance profiles of the *E. coli* isolates included in the seasonal analysis. The detailed statistical output for this analysis can be found in Appendix A13. The counts of MDR *E. coli* did not significantly differ between the sites along the Opara Stream and were consistent over the four seasons, however, all these sites had significantly fewer MDR *E. coli* than the RH site. Across the seven sites, gentamicin resistance was the most observed resistance, followed by ampicillin resistance and ciprofloxacin resistance.

Table 14 Seasonal differences in MDR isolates in Okains Bay.

Season	Total isolates tested	Amp	Chl	Rif	Tet	Kan	Tmp	Gen	Cip	MDR
Winter	117	54%	0%	0%	0%	8%	0%	64%	0%	12%
Spring	93	64%	0%	5%	0%	45%	0%	23%	9%	12%
Autumn	93	1%	0%	2%	0%	0%	5%	0%	75%	8%
Summer	133	5%	0%	5%	2%	0%	0%	32%	0%	7%

4. Discussion

Overall, the results from the antibiotic susceptibility assay indicate that there was significantly fewer MDR *E. coli* in Treated than in Stream. MDR *E. coli* numbers were not significant differently between the three regions. A large proportion of the isolates that were tested were able to transfer resistance genes to other susceptible bacteria, and ESBL- and AmpC-producing *E. coli* were detected in all three water sources.

Selecting for resistant populations using low concentrations of antibiotics was sufficient to isolate MDR *E. coli*, but population densities were high enough that enrichment using low concentrations of antibiotics was not necessary. MDR *E. coli* easily could be identified amongst *E. coli* on TBX without supplemented antibiotics. The largest number of MDR *E coli* was in fact detected from these unenriched cultures.

MDR *E. coli* were not significantly different across the three regions. The results from this chapter further support the fact that total *E. coli* concentrations are not predictive of resistant populations in the environment. This is seen with the *E. coli* from Treated. The numbers of *E coli* from Treated was small, but they had a higher proportion of MDR. This also reinforces the observation that the *E. coli* concentration per se does not correlate with MDR.

In Banks Peninsula, ciprofloxacin resistance was the most observed resistance in the isolates, while ampicillin resistance was the most observed resistance in the other two regions. Ciprofloxacin resistance in the Banks Peninsula is skewed by the sampling event after the COVID-19 lockdown in New Zealand in 2020, gentamicin resistance was the most predominant in Bank peninsula. MDR isolates were observed across the three regions in all water sources. One isolate from the Banks Peninsula region was resistant to all eight antibiotics tested. The Banks Peninsula region was overly represented in my dataset as I had the Okains Bay case study over four seasons. Nevertheless, when controlling for those sampling events, the Banks Peninsula area still had higher overall MDR frequencies.

Cross-resistance between ampicillin and gentamicin and ampicillin and ciprofloxacin was often observed. These cross-resistances were also frequently observed together in the conjugation experiments where ampicillin and gentamicin resistance and ampicillin and ciprofloxacin resistance were the most observed resistances shared together with susceptible bacteria. These results suggests that the genes conferring resistance to ampicillin and gentamicin, and ampicillin and ciprofloxacin resistance may be linked on the same plasmid.

Ampicillin, gentamicin and ciprofloxacin resistance were the most common resistances detected in the *E. coli* isolates. Ampicillin and ciprofloxacin are both used to treat infections in humans, companion animals, and in livestock and gentamicin is used to treat infections in humans and companion animals. In human clinical *Enterobacter* spp. isolates, ampicillin, fluoroquinolone and gentamicin resistance were detected at the highest frequencies (Institute of Environmental Science and Research, 2017a). In the environment, ampicillin and ciprofloxacin resistance are often detected, however, gentamicin resistance is not detected at the same frequencies (Adewale, 2018; Schousboe et al., 2015). No information could be found regarding the use of gentamicin in livestock in New Zealand. The detection of gentamicin resistance at a similar a similar frequency to ampicillin and ciprofloxacin resistance could suggest significant contamination of surface waters from human or companion animal sources. Alternatively, aminoglycosides may have use in other sectors that were not explored in this thesis, such as horticulture, and may be entering into the environment through that sector.

Tetracycline and rifampicin resistance were detected at the highest frequency in Canterbury. Tetracycline is used to treat infections in humans, companion animals and livestock, but rifampicin is predominantly used to treat infections only in humans. Tetracycline resistance in human clinical isolates is not as common as it is in livestock. Tetracycline resistance has previously been detected in high frequencies in pig farms in New Zealand (Nulsen et al., 2008). The pork industry is small in New Zealand. Canterbury is the largest pork producer, producing approximately 60% of all pork products in New Zealand (Statistics New Zealand, 2020a). The pork and poultry sector consume almost half of the antibiotics purchased for use in animals and horticulture annually, with tetracycline being one of the antibiotics used in these sectors (New Zealand Food Safety, 2020). The higher rates of tetracycline resistance observed in the

Canterbury region could be associated with the presence of pork farms. Other studies have found rifampicin resistance at high frequencies in the environment (Ibekwe et al., 2011). Rifampicin resistance is due to a single point mutation in the *rpoB* gene. Resistance can occur at a higher frequency than the other antibiotics explored in this thesis, however, the occurrence of the point mutations still requires the presence of rifampicin. This suggests that there may be rifampicin residues in the environment.

Chloramphenicol and kanamycin resistance were some of the lowest frequency resistances observed. Chloramphenicol and kanamycin resistance are not common in New Zealand. These antibiotics are predominantly used in human medicine, with chloramphenicol not being permitted for use in food producing animals in New Zealand (New Zealand Food Safety, 2018). Adewale (2018) detected chloramphenicol resistance at a higher frequency in the urban Ōtākaro/Avon river, and detected kanamycin resistance in the urban Ōtākaro/Avon River but not in the rural Silverstream. The lower detection of chloramphenicol and kanamycin resistance in rural surface waters could be due to both antibiotics being reserved for human use. Trimethoprim resistance was rare and only detected in Banks Peninsula. In New Zealand, trimethoprim is widely used in humans, companion animals, and in livestock. Trimethoprim resistance in clinical human isolates has been increasing (New Zealand Medicines and Medical Devices Safety Authority, 2018), and resistance in rural environments have been previously detected at low frequencies (Adewale, 2018; Van Hamelsveld et al., 2019). Trimethoprim resistance and ESBL-producing bacteria have been linked in clinical human isolates (Auer et al., 2010; Critchley et al., 2019; Institute of Environmental Science and Research, 2017a; Rodríguez-Baño et al., 2018), however, no association between trimethoprim resistance and ESBL-production was found in my isolates.

ESBL-producing *E. coli* were detected at a lower frequency than AmpC-producing *E. coli*. Ampicillin and ciprofloxacin resistance were not predictive of ESBL production, but ciprofloxacin was predictive of AmpC production. In the tested isolates, ESBL and AmpC production was not correlated with MDR. This result contrasts what is seen in clinical isolates and in the environment, however, these results are only representative of the antibiotics that were tested.

Okains Bay

Season had no impact on MDR *E. coli* counts in Okains Bay. The residential home had significantly more MDR *E. coli* than the sites along the Opara Stream, and MDR *E. coli* were detected in one of the sources of the Opara Stream. This suggests that MDR *E. coli* are contaminating the Opara Stream from the source, however, as this was a one-off sampling event, repeat samples would be needed for conformation. There were no significant differences in MDR *E. coli* numbers across the sites along the Opara Stream. This could mean that there are consistently MDR *E. coli* contaminating the stream from the most upstream site that I sampled.

Different resistance patterns were observed between the four seasons. As Banks Peninsula is a place with concentrated agricultural uses including livestock farming in catchment areas, the differences in the resistance patterns could be associated with different phases in the farming cycle. It could also be due to the changes in tourism as an effect of COVID-19. Gentamicin resistance was the most commonly observed resistance in Okains Bay, followed by ampicillin and ciprofloxacin resistance. As discussed above, this could indicate contamination from humans or companion animals.

3.15 Conclusion

The results from this chapter show that the hypothetical tourist travelling around New Zealand is exposed to MDR bacteria that are able to share their resistance genes through their interaction with surface waters. This supports both my hypotheses for this chapter. The results obtained from the frequency of MDR *E. coli* from the three water sources across the three regions indicate that anthropogenic influence on surface waters may contribute to MDR in the environment. Further, preliminary analysis identified that MDR *E. coli* can be detected in the surface water sources. Seasons had no effect on the frequency of MDR *E. coli.* Further studies will be needed to investigate the associated resistance to all classes of antibiotics in surface waters in New Zealand.

Chapter 4: Investigating the underlying genetic determinants of resistance and conjugation

The rising frequency of antibiotic resistance in human-associated pathogens is a threat to human health and to the productivity of the plants and animals we cultivate for food, fuel and material. To address this threat, we must understand how resistant determinants are evolving. Antibiotic resistance is the outcome of pre-existing antibiotic resistance genes amplified through selection of phenotypes made fit by our own use of antibiotics and other chemical agents (Rupp & Fey, 2003), as discussed in Chapter 1. Bacterial resistance can be intrinsic, adaptive or acquired. Antibiotic resistance genes can be acquired through horizontal gene transfer or through mutations. The acquired resistances may or may not be specific to particular antibiotics. In this chapter, the putative biochemical mechanisms of resistance are diagnosed using *in silico* methods.

4.1 Multi drug resistance efflux transporters and porins

Native efflux pumps in the bacterial membrane have broad substrate specificity and can confer low- and high-level resistance to an array of antibiotics by decreasing drug accumulation within bacteria (Hooper, 2001; Huguet et al., 2013). Mutations in the genes which encode for these membrane efflux systems can actively increase the efflux of antibiotics out of the cell by either deregulating their expression (Alcalde-Rico et al., 2016) or altering their substrate range. Mutations in porins can reduce the initial uptake by reducing channels size (Huguet et al., 2013).

Efflux pumps are grouped into five structural families; the resistance-nodulation-division (RND), small multidrug resistance (SMR), multi antimicrobial extrusion (MATE), major facilitator superfamily (MFS), and the ATP-binding cassette (ABC) superfamilies (Alcalde-Rico et al., 2016). There are many efflux systems in *E. coli*, some of which contribute to multidrug resistance, but the main multidrug drug resistance efflux system in *E. coli* is the AcrAB-TolC efflux system of the RND structural family (Bergmiller et al., 2017; Li et al., 2015; Wen et al., 2018). Mutations in AcrAB-TolC regulatory enzymes such as AcrR and MarR have been shown to increase multidrug resistance (Sadeghi, 2019; Wang et al., 2001). Overexpression of *acrA* and *acrB* is strongly associated with fluoroquinolone and multidrug resistance (Swick et al., 2011).

Other efflux systems such as MdfA of the MFS structural family are clinically relevant as they can confer resistance to structurally unrelated antibiotics (Edgar & Bibi, 1997).

4.2 Point mutations and resistance

Mutations that give rise to a resistant phenotype are due to a complex mix of factors that influence the rate and type of mutation that can occur under antibiotic selective pressure (Martinez & Baquero, 2000). Point mutations in the enzymes that are the targets of antibiotics can lead to a resistant phenotype. For instance, resistance to rifampicin is primarily caused by mutations in the rifampicin resistance determining region (RRDR), an 81bp region in the *rpoB* gene (Goldstein, 2014). These mutations decrease rifampicin's affinity for the binding site of the RNA polymerase. Subtherapeutic doses of rifampicin can select for resistance. This is also seen in ciprofloxacin resistance, where the most commonly associated ciprofloxacin resistance mechanism is through specific mutations in the quinolone resistance-determining region (QRDR) where *gyrA*, *gyrB*, *parC* and *parE* are present (Aldred et al., 2014). These mutations confer resistance by reducing ciprofloxacin's binding affinity to the enzyme-DNA complex (Hooper & Jacoby, 2015; Jacoby, 2005).

As discussed in Chapter 3, ESBL- and AmpC-producing bacteria are important threats to how antibiotics are used to treat infections. ESBL enzymes are derived from TEM-1 and -2 through point mutations that alter the configuration of the active site (Paterson & Bonomo, 2005). AmpC-mediated resistance is predominantly through overexpression of the chromosomal *ampC*. Overexpression of AmpC occurs from mutations in the promoter and attenuator region (Corvec et al., 2002; Tracz et al., 2005). Point mutations in the bacterial genome can have serious implications for the effectiveness of the antibiotic we use. Through whole genome sequencing, we can further understand the importance of mutations and the role that they play in antibiotic resistance.

4.3 Plasmids (sequence types)

Plasmid-mediated conjugation is one of the principal routes that aids the dissemination of antibiotic resistance genes (Wozniak & Waldor, 2010). To that end, databases to identify known

plasmid types have been developed to assist in the rapid detection of MDR Enterobacteriaceae from raw sequence data or contigs (Carattoli et al., 2014). The PlasmidFinder database is able to detect replicons in WGS and assign incompatibility (Inc) groups, while the pMLST Web tool is able to subtype plasmids, providing information about plasmid sequence types (STs) and any new variants or alleles. The information from these two databases provides an insight into possible lineage of plasmids on a global scale.

Plasmids with Inc groups IncF, IncI, IncA/C, IncL, IncN and IncH have been reported as having the widest variety of resistance genes in Enterobacteriaceae (Rozwandowicz et al., 2018). The plasmid group IncA/C has been associated with resistance to aminoglycosides, β-lactams, chloramphenicol, tetracyclines and trimethoprim (Hoffmann et al., 2017), while other plasmid groups such as IncF, IncI and IncN have been identified as early carriers of β-lactam resistance genes (Tran-Dien et al., 2018). IncF, IncI, IncX and IncN plasmid groups have a narrow host range, and are usually found in Enterobacteriaceae (Musovic et al., 2006).

4.4 Aims

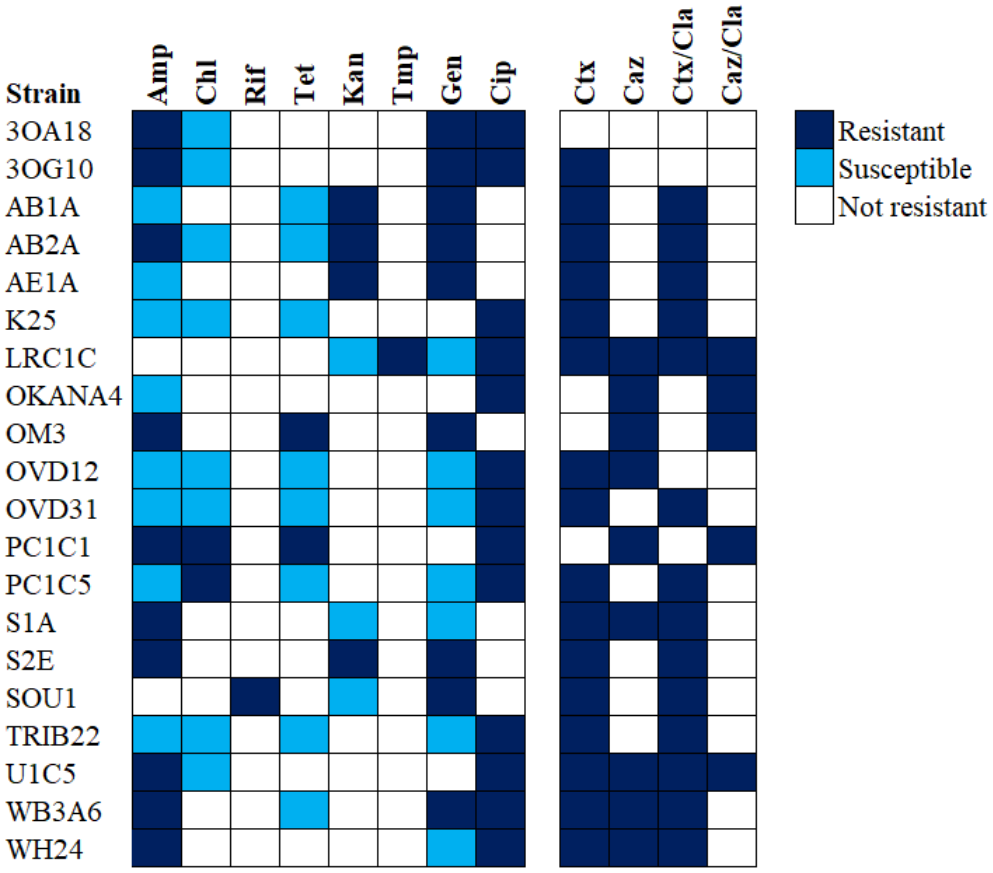
As mutations in the regulatory enzymes AcrR and MarR have been shown to increase multidrug resistance (Sadeghi, 2019; Wang et al., 2001), I wanted to explore whether the known point mutations could be identified in my isolates. I also wanted to observe whether mutations detected in the QRDR were predictive of ciprofloxacin resistance. Similarly, I wanted to investigate whether mutations in the AmpC promoter were predictive of the AmpC production phenotype.

Methods

4.5 Strain selection

20 isolates were selected for sequencing. Isolates were chosen for sequencing based on geographic criteria, resistance profiles or whether they were an ESBL or AmpC producer. The phenotype of the selected strains can be found in (Table 15).

Table 15 Phenotype of the strains selected for sequencing.



Whole genome sequencing (WGS) was done in collaboration with Dr Gayle Ferguson at Massey University in Auckland. Isolates were sequenced by Illumina MiSeq paired-end sequencing. Genomic DNA was prepared for both sequencing platforms using a Wizard Genomic DNA Purification Kit (Promega Corporation) according to the manufacturer's instructions. DNA concentration and purity were quantified using a Qubit Fluorometer (Invitrogen). Raw forward

and reverse sequences were uploaded to the Australian Galaxy server and the genome was assembled with Galaxy SPAdes genome assembler (Galaxy v3.12.0+galaxy1; (Afgan et al., 2018), with parameters: no error correction, automatically chosen k-mer value and no coverage cut off. The genome was annotated in RAST version 2.0 (Aziz et al., 2008) using default settings (RASTtk annotation scheme, automatically fix errors, verbose level = 0). *E. coli* K12 MG1655 (NCBI Accession number: U00096) was used as a reference strain for all analyses (Blattner et al., 1997).

4.6 *E. coli* classification

All *E. coli* isolates were serotyped using SerotypeFinder 2.0 v2.0.1 (Joensen et al., 2015). The threshold for sequence identity was 85%, and the minimum number of nucleotides a sequence must overlap was 65%. PathogenFinder v1.1 (Cosentino et al., 2013) was used to predict whether the *E. coli* isolates were human pathogens. Multi-Locus Sequence Typing (MLST) was performed using MLST 2.0 v2.0.4 (Larsen et al., 2012). Two MLST configurations were available for *E. coli*; Escherichiacoli#1, also known as Achtman configuration (Jolley et al., 2018; Wirth et al., 2006), and Escherichiacoli#2, also known as Pasteur configuration (Jaureguy et al., 2008; Jolley et al., 2018).

4.7 Antibiotic Resistance Genotyping

ResFinder 4.1 (Bortolaia et al., 2020) was used to identify resistance genes. The PointFinder software (Zankari et al., 2017) was selected to be included in the analysis. This was to identify point mutations that could lead to resistance. For chromosomal point mutations, the threshold for sequence identity was 90%, and the minimum number of nucleotides a sequence must overlap was 60%. Identification of unknown mutations that are not found in the database was selected. Identification of acquired antimicrobial resistance genes was also selected to be a part of the analysis. All antimicrobial gene databases available were used. The threshold for sequence identity was 90%, and the minimum number of nucleotides a sequence must overlap was 60%. ClustalW Multiple Alignment (Thompson et al., 1994) with 1000 bootstrap replicates was used to compare sequences of genes of interest across the isolates and the reference strain.

4.8 Plasmid Typing and Mobile Genetic Elements

PlasmidFinder 2.1 v2.0.1 was used for plasmid detection (Carattoli et al., 2014). The threshold for sequence identity was 95%, and the minimum number of nucleotides a sequence must overlap was 60%. Plasmid Multi-Locus Sequence Typing (pMLST) was performed using pMLST 2.0 v0.1.0 (Carattoli et al., 2014). Two configurations were explored: IncF RST and IncI1 MSLT. Mobile Element Finder v1.0.3 (Johansson et al., 2020) was used to identify mobile genetic elements and their relation to antibiotic resistance and virulence factors. Acquired resistance genes and virulence gene databases were included in the analysis.

4.9 Statistics

I wanted to observe whether the mutations detected in the QRDR were predictive of ciprofloxacin resistance. I also wanted to investigate whether mutations in the AmpC promoter were predictive of the AmpC production phenotype. For both analyses, a generalized linear model (GLM) with a binomial link was selected.

Results

All strains were predicted to be human pathogens. This was due to the isolates harbouring genes that were associated with other known *E. coli* pathogens. Appendix D describes the PathogenFinder results, as well as the SerotypeFinder and MLST results. There was evidence of putative macrolide, lincosamide and streptogramin B resistance, as well as unknown macrolide, aminoglycoside, tetracycline, fluoroquinolone, phenicol and rifamycin resistance genes in all sequenced isolates. Strains OKANA4 and OM3 had predicted nalidixic acid and ciprofloxacin resistance genes. Disinfectant resistance was predicted for strains OM3, PC1C1 and S1A. All but two strains harboured mutations in the *pmrA* and *pmrB* genes. These are associated with colistin resistance (Table 16). Other point mutations of significance were also detected; however, the phenotypes were unknown. This information can be found in Appendix E1. Multidrug transporter *mdfA* was identified as a contributor to resistance for all strains except *E. coli* OM3. The *mdfA* genes from nine isolates were identified as being on a mobile genetic element associated with the IS families IS200/IS605 (n = 5) and IS630 (n = 8).

Point mutations in the QRDR that are known to confer resistance to ciprofloxacin were detected in my isolates, however, there was no significant effect of these point mutations on the ciprofloxacin resistance phenotypes (Table 16). Other point mutations of significance were also detected, however, the phenotypes were unknown. This information can be found in Appendix E2. The multidrug efflux transport system *emrE* was identified in 11 strains. Point mutations in genes associated with colistin resistance were identified in 19 strains.

No genes associated with ESBL-production were identified. The *blaEC* (allelic to *amp*C) detections in the isolates were not a perfect match to the reference strain *E. coli* K12 MG 1655. 16 alleles of *blaEC* were detected. Point mutations in the *ampC* promoter sequence that have been associated with AmpC overexpression were identified, however, they had no significant effect on AmpC production in my isolates (Table 16). Other point mutations of significance were also detected; however, the phenotypes were unknown. This information can be found in Appendix E1.

The strains OKANA4 and TRIBB22 both harboured the T213I and N214T alleles of *acrR* which have been associated with overexpression of the AcrAB-TolC efflux system. Strain OM3 appeared to have a 2bp insertion at the end of the *acrR* sequence. Three isolates had deletions either at the beginning or the end of the *marR* gene. All isolates harbored the G103S point mutation allele, and 17 isolates harboured the Y137H point mutation allele. These mutations have been identified as leading to overexpression of the AcrAB-TolC efflux system, contributing to MDR. Other point mutations also were detected in *marR*. A sequence alignment of *marR* across the isolates can be seen in Figure 10.

Plasmids were detected in 11 isolates. The most common plasmid incompatibility group detected by sequencing was IncF (n = 13). IncI (n = 2) and IncX (n = 2) incompatibility groups were also detected. Five isolates had more than one plasmid. Six isolates with plasmids were conjugative donors as shown in Chapter 3. One *E. coli* strain harboured a likely non-conjugative plasmid because it generated transconjugants at an undetectable frequency. Because the isolates were sequenced using the Illumina sequencing platform which generates short reads, I was unable to construct the plasmids from the contig sequences.

Table 16 Point mutations in the sequenced isolates that have been identified as contributing to a resistant phenotype. *ampC* promoter point mutations that are associated with overexpression. *gyrA*, *gyrB*, *parC* and *parE* mutations that are associated with ciprofloxacin resistance. *pmrA* and *pmrB* point mutations that are associated with colistin resistance. **E. coli* K12 used as a reference strain.

	ampC		*gyrA*		*gyrB*					*parC*					*parE*			*pmrA*			*pmrB*			
Strains	**-1C>T**	**-18G>A**	**D678E**	**A828S**	**E185D**	**R206K**	**S492N**	**A618T**	**E703D**	**S57T**	**E62K**	**D475E**	**Q481L**	**T718A**	**V136I**	**I355T**	**D475E**	**T31S**	**I128N**	**G144S**	**H2R**	**E123D**	**D283G**	**V351I**
*K12											■													
3OA18			■						■		■										■		■	
3OG10			■						■		■										■		■	
AB1A			■	■	■						■	■			■			■	■	■	■	■	■	■
AB2A	■	■									■												■	
AE1A			■								■										■		■	
K25			■	■			■	■			■		■	■	■		■	■			■	■	■	■
LRC1C	■	■	■								■													
OKANA4										■	■	■								■	■		■	
OM3			■			■				■	■				■	■			■		■		■	■
OVD12	■	■									■												■	
OVD31	■	■									■												■	
PC1C1			■	■				■			■	■			■			■	■	■	■	■	■	■
PC1C5			■								■	■									■		■	
S1A			■	■							■	■			■			■	■	■	■	■	■	■
S2E			■						■		■										■		■	
SOU1			■	■							■	■		■	■			■	■	■	■	■	■	■
TRIB22											■									■	■		■	
UC15			■	■			■	■			■	■	■	■	■		■	■			■	■	■	■
WB3A6	■	■									■												■	
WH24			■	■	■						■	■		■	■			■	■	■	■	■	■	■

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                    5         15         25         35         45         55         65         75         85         95        105        115        125        135
3OA18     MKSTSDLFNE IIPLGRLIHM VNQKKDRLLN EYLSPLDITA AQFKVLCSIR CAACITPVEL KKVLSVDLGA LTRMLDRLVC KGWVERLPNP NDKRGVLVKL TTSGAAICEQ CHQLVGQDLH QELTKNLTAD EVATLEHLLK KVLP
3OG10     MKSTSDLFNE IIPLGRLIHM VNQKKDRLLN EYLSPLDITA AQFKVLCSIR CAACITPVEL KKVLSVDLGA LTRMLDRLVC KGWVERLPNP NDKRGVLVKL TTSGAAICEQ CHQLVGQDLH QELTKNLTAD EVATLEHLLK KVLP
AB1A      MKSTSDLFNE IIPLGRLIHM VNQKKDRLLN EYLSPLDITA AQFKVLCSIR CAACITPVEL KKVLSVDLGA LTRMLDRLVC KGWVERLPNP NDKRGVLVKL TTSGAAICEQ CHQLVGQDLH QELTKNLTAD EVATLEHLLK KVLP
AB2A      MKSTSDLFNE IIPLGRLIHM VNQKKDRLLN EYLSPLDITA AQFKVLCSIR CAACITPVEL KKVLSVDLGA LTRMLDRLVC KGWVERLPNP NDKRGVLVKL TTSGAAICEQ CHQLVGQDLH QELTKNLTAD EVATLEHLLK KVLP
AE1A      MKSTSDLFNE IIPLGRLIHM VNQKKDRLLN EYLSPLDITA AQFKVLCSIR CAACITPVEL KKVLSVDLGA LTRMLDRLVC KGWVERLPNP NDKRGVLVKL TTSGAAICEQ CHQLVGQDLH QELTKNLTAD EVATLEHLLK KVLP
K_12*     MKSTSDLFNE IIPLGRLIHM VNQKKDRLLN EYLSPLDITA AQFKVLCSIR CAACITPVEL KKVLSVDLGA LTRMLDRLVC KGWVERLPNP NDKRGVLVKL TTGGAAICEQ CHQLVGQDLH QELTKNLTAD EVATLEYLLK KVLP
K25       MKSTSDLFNE IIPLGRLIHM VNQKKDRLLN EYLSPLDITA AQFKVLCSIR CAACITPVEL KKVLSVDLGA LTRMLDRLVC KGWVERLPNP NDKRGVLVKL TPSGAAICEQ CHQLVGQDLH Q--------- ---------- ----
OKANA4    MKSTSDLFNE IIPLGRLIHM VNQKKDRLLN EYLSPLDITA AQFKVLCSIR CAACITPVEL KKVLSVDLGA LTRMLDRLVC KGWVERLPNP NDKRGVLVKL TTSGAAICEQ CHQLVGQDLH QELTKNLTAD EVATLEHLLK KVLP
OM3       MKSTSDLFNE IIPLGRLIYM VNQKKDRLLN DYLSPLDITA AQFKVLCSIR CAACITPVEL KKVLSVDLGA LTRMLDRLVC KGWVERLPNP NDKRGVLVKL TASGAAICEQ CHQLVGQDLH QELTKNLTAD EVATLEHLLK KVLP
OVD12     MKSTSDLFNE IIPLGRLIHM VNQKKDRLLN EYLSPLDITA AQFKVLCSIR CAACITPVEL KKVLSVDLGA LTRMLDRLVC KGWVERLPNP NDKRGVLVKL TTSGAAICEQ CHQLVGQDLH QELTKNLTAD EVATLEHLLK KVLP
OVD31     MKSTSDLFNE IIPLGRLIHM VNQKKDRLLN EYLSPLDITA AQFKVLCSIR CAACITPVEL KKVLSVDLGA LTRMLDRLVC KGWVERLPNP NDKRGVLVKL TTSGAAICEQ CHQLVGQDLH QELTKNLTAD EVATLEHLLK KVLP
PC1C1     MKSTSDLFNE IIPLGRLIHM VNQKKDRLLN EYLSPLDITA AQFKVLCSIR CAACITPVEL KKVLSVDLGA LTRMLDRLVC KGWVERLPNP NDKRGVLVKL TTSGAAICEQ CHQLVGQDLH ---------- ---------- ----
PC1C5     MKSTSDLFNE IIPLGRLIHM VNQKKDRLLN EYLSPLDITA AQFKVLCSIR CAACITPVEL KKVLSVDLGA LTRMLDRLVC KGWVERLPNP NDKRGVLVKL TTSGAAICEQ CHQLINKKPD GGRSGNT--- ---------- ----
S1A       MKSTSDLFNE IIPLGRLIHM VNQKKDRLLN EYLSPLDITA AQFKVLCSIR CAACITPVEL KKVLSVDLGA LTRMLDRLVC KGWVERLPNP NDKRGVLVKL TTSGAAICEQ CHQLVGQDLH QELTKNLTAD EVATLEHLLK KVLP
S2E       MKSTSDLFNE IIPLGRLIHM VNQKKDRLLN EYLSPLDITA AQFKVLCSIR CAACITPVEL KKVLSVDLGA LTRMLDRLVC KGWVERLPNP NDKRGVLVKL TTSGAAICEQ CHQLVGQDLH QELTKNLTAD EVATLEHLLK KVLP
SOU1      MKSTSDLFNE IIPLGRLIHM VNQKKDRLLN EYLSPLDITA AQFKVLCSIR CAACITPVEL KKVLSVDLGA LTRMLDRLVC KGWVERLPNP NDKRGVLVKL TTSGAAICEQ CHQLVGQDLH QELTKNLTAD EVATLEHLLK KVLP
TRIB22    MKSTSDLFNE IIPLGRLIHM VNQKKDRLLN EYLSPLDITA AQFKVLCSIR CAACITPVEL KKVLSVDLGA LTRMLDRLVC KGWVERLPNP NDKRGVLVKL TTSGAAICEQ CHQLVGQDLH QELTKNLTAD EVATLEHLLK KVLP
UC15      ---------- ---------- ---------- ---------- ----MLCSIR CAACITPVEL KKVLSVDLGA LTRMLDRLVC KGWVERLPNP NDKRGVLVKL TPSGAAICEQ CHQLVGQDLH QELTKNLTAD EVATLEHLLK KVLP
WB3A6     MKSTSDLFNE IIPLGRLIHM VNQKKDRLLN EYLSPLDITA AQFKVLCSIR CAACITPVEL KKVLSVDLGA LTRMLDRLVC KGWVERLPNP NDKRGVLVKL TTSGAAICEQ CHQLVGQDLH QELTKNLTAD EVATLEHLLK KVLP
WH24      MKSTSDLFNE IIPLGRLIHM VNQKKDRLLN EYLSPLDITA AQFKVLCSIR CAACITPVEL KKVLSVDLGA LTRMLDRLVC KGWVERLPNP NDKRGVLVKL TTSGAAICEQ CHQLVGQDLH QELTKNLTAD EVATLEHLLK KVLP
LRC1C     MKSTSDLFNE IIPLGRLIHM VNQKKDRLLN EYLSPLDITA AQFKVLCSIR CAACITPVEL KKVLSVDLGA LTRMLDRLVC KGWVERLPNP NDKRGVLVKL TTSGAAICEQ CHQLVGQDLH QELTKNLTAD EVATLEHLLK KVLP
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Figure 10 ClustalW alignment of *marR* across the sequenced isolates. Point mutations in *marR* that have been associated with overexpression of the AcrAB-TolC efflux system highlighted by the blue boxes. *Reference strain *E. coli* K12

Table 17 Results from PlasmidFinder and plasmid replicon database. *Novel allele detected, ST may indicate nearest ST; ? Imperfect hit, ST cannot be trusted; - No hits.

***E. coli* strain**	**Conjugative phenotype**	**PlasmidFinder**	**pMLST IncF RST**	**pMLST IncI1 MLST**
3OBA18	Not tested	IncFIA(HI1)	ST F^{-}:A^{-}:B^{-}	-
3OG10	Not tested	-	ST F^{-}:A^{-}:B^{-}	-
AB1A	Ampicillin	IncFII IncFIA(HI1)	ST F25:A^{-}:B^{-}	-
AB2A	Ampicillin, chloramphenicol	IncI(Gamma)	-	ST Unknown*
AE1A	-	-	-	-
K25	-	-	-	-
LRC1C	-	-	ST F74:A-:B45	-
OKANA4	-	IncFIB(AP001918) IncFIB(H89-PhagePlasmid)	ST F19:A-:B-?	-
OM3	Not tested	IncFIB(AP001918) IncFIC(FII)	ST F89:A-:B54;	-
OVD12	Ampicillin, ciprofloxacin, chloramphenicol	IncFIB(AP001918)	ST F112:A-:B-	-
OVD31	Ampicillin, chloramphenicol	IncFIB(AP001918)	ST F112:A-:B-	-
PC1C1	-	-	ST F-:A-:B-	-
PC1C5	-	-	ST F35:A-:B-	ST Unknown*
S1A	Not tested	-	ST F-:A-:B-	-
S2E	-	-	St F-:A-:B-	-
SOU1	Not tested	IncFIB(AP001918)	ST F-:A-:B24	-
TRIB22	Ampicillin, gentamicin	IncFII(pHN7A8) IncX1 IncX4	ST F40:A-:B- ;	-
UC15	-	-	No hits	-
WB3A6	Not tested	IncI1_1_Alpha_AP005147 IncFIB(pB171) pB171 - blaNDM-5	ST F57:A-:B23	ST Unknown
WH24	Gentamicin, ciprofloxacin	IncFIA(HI1)	-	-

Discussion

Here I described 20 environmental *E. coli* isolates with various genotypes associated with antibiotic resistance. Overall, the phenotype was congruent with genotype. Based on the genotype information, AmpC overexpression, point mutations in target genes, and efflux systems are likely significant contributors to the MDR phenotypes.

In *E. coli*, AmpC-mediated resistance is predominantly through overexpression of the chromosomal *ampC*, however, plasmid-mediated *ampC* can also confer resistance. AmpC is constitutively expressed in *E. coli* as it lacks the *ampR* regulator gene (Corvec et al., 2002). Overexpression of AmpC occurs from mutations in the AmpC promoter and attenuator regions which are thought to generate promoters that more closely resemble the *E. coli* consensus sequence (Corvec et al., 2002; Tracz et al., 2005). Previous studies have identified a pair of mutations in the *ampC* promoter that leads to increased expression. In my isolates, the pair was always identified together in a genome and was only identified in six strains even though 18 strains displayed a AmpC-producing phenotype. Strains that harboured this pair were generally more resistant to cefotaxime or ceftazidime with the exception of strain OVD31. This suggests that these mutations are contributors to AmpC production, which is supported by other findings (Nelson & Elisha, 1999; Siu et al., 2003). There were 16 allelic variants of *blaEC* identified in my study, and none were identical to the reference *E. coli* K12 MG1655 or known $_{bla}$*EC* sequences that were available on the NCBI database ($_{bla}$*EC-5*, $_{bla}$*EC-8*, $_{bla}$*EC-13*, $_{bla}$*EC-14*, $_{bla}$*EC-15*, $_{bla}$*EC-16*, $_{bla}$*EC-18* and $_{bla}$*EC-19)*. Another common mutation I found in the promoter sequence was $^{-}$28G>A (n = 5), however, this mutation had not been identified in the literature. The various AmpC and ESBL phenotypes could be attributed to these variations in *blaEC.* It could also be due to a combination of AmpC production and efflux systems.

Fluoroquinolone resistance occurs through three main mechanisms; through mutations the gene sequences for DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*), through mutations that alter cell permeability, and through the acquisition of plasmids harbouring resistance genes (Hooper & Jacoby, 2015). The most commonly associated ciprofloxacin resistance mechanism is through specific mutations in the quinolone resistance-determining

region (QRDR) where *gyrA*, *gyrB*, *parC* and *parE* are present (Aldred et al., 2014). These mutations confer resistance by reducing ciprofloxacin's binding affinity to the enzyme-DNA complex (Hooper & Jacoby, 2015; Jacoby, 2005). Various studies have discovered different amino acid substitutions at various locations within each gene that confer resistance to ciprofloxacin, however, the congruent finding is that a mutation in either DNA gyrase or topoisomerase IV alone is enough to increase ciprofloxacin resistance (Aldred et al., 2014; Heisig, 1996; Mbelle et al., 2019; Zhang et al., 2018). The presence of known point mutations in the QRDR that confer resistance to ciprofloxacin could not be significantly correlated with ciprofloxacin resistance in my isolates. The strongest predictor of a ciprofloxacin resistant phenotype was the *gyrA* D678E point mutation. There were many point mutations of significance that were detected in genes in the QRDR, however, most of them led to an unknown phenotype and had not been identified in the literature before. Sadeghi (2019) showed that *E. coli* with mutations in *acrR* and *marR* had higher ciprofloxacin MICs. 66% of the ciprofloxacin resistant isolates that had an MIC of 2μg mL^{-1} had large deletions in *marR*. Ciprofloxacin resistance in these isolates was likely due to a combination of mutations in efflux systems and the mutations in the target genes the QRDR. Colistin resistance was not something I tested for in my antibiotic susceptibility assay, however, PointFinder detected mutations in the *pmrA* and *pmrB* genes that have been associated with colistin resistance. There were other point mutations of significance that were detected in those genes that had unknown phenotype and had not been identified in the literature before.

Studies have shown increased susceptibility to some antibiotics in strains that have the AcrAB-TolC efflux system knocked out, showing that it is a contributor to resistance (Kurenbach et al., 2017; Li et al., 2015; Sadeghi, 2019; Wang et al., 2001). Further, mutations in the repressors *marR* and *acrR* have been shown to lead to overexpression of the AcrAB-TolC efflux system, further contributing to antibiotic resistance. Point mutations that have been identified before by Sadeghi (2019) and Wang et al. (2001) were detected in the sequenced isolates. These mutations have been linked to increased fluoroquinolone and multidrug resistance. Overexpression of AcrAB-TolC has been shown to increase resistance to five of the antibiotics that I investigated (ampicillin, chloramphenicol, trimethoprim, kanamycin and ciprofloxacin) (Li et al., 2015). Two of the strains I isolated were conjugative donors and had *mdfA* on a mobile genetic element.

Unfortunately, plasmid sequences were unable to be extracted from the contigs, which hindered the ability to observe for plasmid-mediated resistant determinants. The multidrug efflux system EmrE was identified in 11 isolates. EmrE has been shown to confer resistance to aminoglycosides and is part of the SMR family (Nasie et al., 2012). Multidrug efflux transporters have been widely recognized as contributing to antibiotic resistance. *E. coli* have many multidrug efflux systems which contribute to resistance. AcrAB-TolC and MdfA were explored because AcrAB-TolC had been identified as being the key multidrug efflux system in *E. coli* and MdfA was predicted to be a contributor or resistance by ResFinder 4.1.

4.10 Conclusions

The key findings are that a combination of point mutations, efflux systems and AmpC β–lactamases are the putative contributors to resistance phenotypes observed in the sequenced isolates. The contamination of antibiotic residues and other selective agents into surface waters can lead to adaptive responses in bacteria (Kurenbach et al., 2015; Lupo et al., 2012). Through WGS we can identify the underlying genetic mechanisms that confer resistance, and further understand how the environment plays a role in antibiotic resistance.

4.11 Final summary and future perspectives

The story of the journey I describe begins with airplane water and flows all the way to WGS *E. coli* isolates. The key conclusions drawn from my thesis are that:

1) *E. coli* were below the detection limit in both long haul and domestic airplane water, however, long haul planes had significantly higher microbial loads than domestic planes. As a species profile was not done for these isolates, a conclusion cannot be drawn on whether they were pathogenic or not. The overarching conclusion, however, is that passengers on long haul planes have opportunity to be exposed to water of poorer microbiological quality.

2) Treating raw water from streams significantly reduces the concentration of *E. coli* that we may be exposed to, however, as total *E. coli* concentrations are not predictive of resistant populations, the risk of being of exposed to a MDR *E. coli* is not reduced proportionally.

3) MDR *E. coli* can be detected across the South Island of New Zealand. These isolates were resistant to antibiotics that are classed by the WHO as 'critical' or 'highly important' for human therapeutics. No significant difference in MDR counts were found between regions. Each region had different resistance profiles, which in some cases aligned with the anthropogenic activities that are dominant in those places.

4) MDR *E. coli* were able to share their resistant determinants through horizontal gene transfer, and for some isolates, at a high frequency (1×10^{-1} cfu mL^{-1}). ESBL- and AmpC-producing *E. coli* were also detected.

5) Genetic analysis showed that point mutations, efflux systems and AmpC-production were like significant contributors to multidrug resistance.

The study emphasized geographic range over replication depth. A limitation of this was that I was able to sample from many places, however, this meant that repeat samples were not always feasible to obtain from sites of interest. Further work could build on these results, focusing on areas where MDR *E. coli* were detected and investigating stronger links to land use patterns and MDR bacteria. Seasonal sampling of Okains Bay provided a glimpse into the different resistance profiles relative to different seasons. Further seasonal sampling would be beneficial to observe whether these resistance patterns are consistent, especially with the identification of ciprofloxacin resistant populations in autumn. Further analysis could observe whether the ESBL- and AmpC-producing genetic determinants can be shared through horizontal gene transfer. This would be an important analysis as ESBL- and AmpC-producing *E. coli* are an important threat to how antibiotics are used to treat infections and determining whether they can be disseminated in these surface waters would provide an insight into another possible vector of exposure.

The isolates which were able to share resistant determinants at a high frequency could be sequenced. It would be interesting to observe the underling features that result in this observation. Mutations associated with colistin resistance were detected in some of the sequenced isolates. Further study could investigate the presence of colistin resistance in the environment. Colistin is an important last resort antibiotic for carbapenem-resistant

Enterobacteriaceae infections (Gharaibeh & Shatnawi, 2019), and determining the presence of colistin resistance genes in the New Zealand environment would be important endeavour. Based on my genotype results, AmpC overexpression, point mutations in target genes, and efflux systems are likely significant contributors to the MDR phenotypes. Further analysis could investigate the contribution of efflux systems and these point mutations to levels of resistance in environmental isolates.

Antibiotic resistance is a global crisis, and mortalities associated with antibiotic resistant infections are predicted to increase to over 10 million by 2050 (Tagliabue & Rappuoli, 2018). Though New Zealand may have low incidences of antibiotic resistance, a rising trend of antibiotic resistant infections has been observed (Ministry of Health & Ministry for Primary Industries, 2017a). Recognising the contribution to the antibiotic 'resistome' through our anthropogenic activities is an integral part of antibiotic stewardship and to protect the future use of antibiotics.

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Appendices

Appendix A: Statistical outputs for water quality and multidrug resistance analyses

A1 Comparison of long haul vs short haul airplane water

Mesophile counts between airplane water using glm.

glm(formula = mesophile_per_100uL ~ haul, family = poisson(link = "log"), data = airplane)

Coefficients:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	5.09222	0.03919	129.926	< 2e-16 ***
hauloverseas	0.32389	0.05145	6.295	3.07e-10 ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Emmeans post hoc analysis:

Contrasts	Estimate	Std. Error	z ratio	p value
domestic - overseas	-0.324	0. 0515	-6.295	<.0001

A2 Comparison of *E. coli* counts

***E. coli* counts between drinking water sources using glmer.**

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)
Family: poisson (log)
Formula: ecoli ~ source + (1 | site) + (1 | total2$obs_effect)
Data: total2

Random effects

Groups	Name	Variance	Std. Dev
total2$obs_effect	(Intercept)	0.02077	0.1441
site	(Intercept)	9.65398	3.1071

Number of obs: 36, groups: total2$obs_effect, 36; site, 12

Fixed effects:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	1.347	1.790	0.752	0.4518
sourceStream	3.087	2.371	1.302	0.1929
sourceTreated	-6.091	2.628	-2.318	0.0205 *

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

	(Intr)	srcStr
sourceStrem	-0.755	
sourceTretd	-0.583	0.440

Emmeans post hoc analysis:

Contrasts	Estimate	Std. Error	z ratio	p value
Other – Stream	-3.09	2.37	-1.302	0.3940
Other – Treated	6.09	2.63	2.318	0.0534
Stream - Treated	9.18	2.65	3.458	0.0016

E. coli counts between and within regions using glmer.

Generalized linear mixed model fit by maximum likelihood (Adaptive Gauss-Hermite Quadrature, nAGQ = 0)
Family: poisson (log)
Formula: ecoli ~ source * region + (1 | site) + (1 | mixed$obs_effect)
Data: mixed

Random effects

Groups	Name	Variance	Std. Dev
total2$obs_effect	(Intercept)	0.02076	0.1441
site	(Intercept)	3.53889	1.8812

Number of obs: 36, groups: mixed$obs_effect, 36; site, 12

Fixed effects:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	5.482	1.883	2.911	0.00361 **
sourceStream	-1.008	2.307	-0.437	0.66225
sourceTreated	-26.784	14794.141	-0.002	0.99856
regionCant	-26.787	14792.943	-0.002	0.99856
regionMarl	-3.118	2.354	-1.325	0.18532
sourceStream:regionCant	28.352	14792.943	0.002	0.99847
sourceTreated:regionCant	46.070	20921.228	0.002	0.99824
sourceStream:regionMarl	1.416	3.299	0.429	0.66768
sourceTreated:regionMarl	3.118	20922.074	0.000	0.99988

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

	(Intr)	srcStr	srcTrt	rgnCnt	rgnMrl	srcS:C	srcT:C	srcS:M
sourceStrem	-0.816							
sourceTretd	0.000	0.000						
regionCant	0.000	0.000	0.000					
regionMarl	-0.800	0.653	0.000	0.000				
srcStrm:rgC	0.000	0.000	0.000	-1.000	0.000			
srcTrtd:rgC	0.000	0.000	-0.707	-0.707	0.000	0.707		
srcStrm:rgM	0.571	-0.699	0.000	0.000	-0.714	0.000	0.000	
srcTrtd:rgM	0.000	0.000	-0.707	0.000	0.000	0.000	0.500	0.000

Emmeans post hoc analysis: only p values less than 0.10 shown.

Contrasts	Estimate	Std. Error	z ratio	p value
Other Banks - Treated Cant	$7.50e^{+00}$	2.57	2.914	0.0853
Stream Banks - Treated Cant	$6.49e^{+00}$	2.20	2.948	0.0778
Stream Cant - Treated Cant	$8.06e^{+00}$	2.57	3.131	0.0458

A3 Comparison of ampicillin resistant *E. coli* populations

Ampicillin resistant *E. coli* in drinking water sources using glmer.

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)
Family: poisson (log)
Formula: amp ~ source + (1 | site) + (1 | amp_obs)
Data: ampresistance

Random effects

Groups	Name	Variance	Std. Dev
amp_obs	(Intercept)	0.2363	0.4861
site	(Intercept)	6.7186	2.5920

Number of obs: 36, groups: amp_obs, 36; site, 12

Fixed effects:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	0.4023	1.5305	0.263	0.7927
sourceStream	1.9184	2.0115	0.954	0.3402
sourceTreated	-4.2604	2.2396	-1.902	0.0571

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

	(Intr)	srcStr
sourceStrem	-0.757	
sourceTretd	-0.562	0.429

Emmeans post hoc analysis:

Contrasts	Estimate	Std. Error	z ratio	p value
Other - Stream	-1.92	2.01	-0.954	0.6062
Other - Treated	4.26	2.24	1.902	0.1380
Stream - Treated	6.18	2.28	2.711	0.0184

Ampicillin resistant *E. coli* between and within regions using glmer.

Generalized linear mixed model fit by maximum likelihood (Adaptive Gauss-Hermite Quadrature, nAGQ = 0)
Family: poisson (log)
Formula: amp ~ source * region + (1 | site) + (1 | mixed$obs_effect)
Data: mixed

Random effects:

Groups	Name	Variance	Std. Dev
total2$obs_effect	(Intercept)	0.2300	0.4795
site	(Intercept)	0.9755	0.9877

Number of obs: 36, groups: mixed$obs_effect, 36; site, 12

Fixed effects:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	$4.536e^{+00}$	$1.028e^{+00}$	4.414	$1.01e^{-05}$ ***
sourceStream	$-1.039e^{+00}$	$1.260e^{+00}$	-0.824	0.40979
sourceTreated	$-2.584e^{+01}$	$1.479e^{+04}$	-0.002	0.99861
regionCant	$-2.584e^{+01}$	$1.479e^{+04}$	-0.002	0.99861
regionMarl	$-3.459e^{+00}$	$1.307e^{+00}$	-2.646	0.00815 **
sourceStream:regionCant	$2.481e^{+01}$	$1.479e^{+04}$	0.002	0.99866
sourceTreated:regionCant	$4.590e^{+01}$	$2.092e^{+04}$	0.002	0.99825
sourceStream:regionMarl	$-3.767e^{-02}$	$1.904e^{+00}$	-0.020	0.98422
sourceTreated:regionMarl	$3.459e^{+00}$	$2.092e^{+04}$	0.000	0.99987

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

	(Intr)	srcStr	srcTrt	rgnCnt	rgnMrl	srcS:C	srcT:C	srcS:M
sourceStrem	-0.815							
sourceTretd	0.000	0.000						
regionCant	0.000	0.000	0.000					
regionMarl	-0.786	0.641	0.000	0.000				
srcStrm:rgC	0.000	0.000	0.000	-1.000	0.000			
srcTrtd:rgC	0.000	0.000	-0.707	-0.707	0.000	0.707		
srcStrm:rgM	0.540	-0.662	0.000	0.000	-0.687	0.000	0.000	
srcTrtd:rgM	0.000	0.000	-0.707	0.000	0.000	0.000	0.500	0.000

Emmeans post hoc analysis: only p values less than 0.10 shown.

Contrasts	Estimate	Std. Error	z ratio	p value
Other Banks - Treated Cant	$5.77e^{+00}$	1.47	3.936	0.0027
Other Banks - Stream Marl	$4.54e^{+00}$	1.56	2.903	0.0879
Stream Banks - Treated Cant	$4.74e^{+00}$	1.28	3.711	0.0064

A4 Comparison of ciprofloxacin resistant *E. coli* populations

Ciprofloxacin resistant *E. coli* in drinking water sources analysis using glmer.

Generalized linear mixed model fit by maximum likelihood (Adaptive Gauss-Hermite Quadrature, nAGQ = 0)
Family: binomial (logit)
Formula: cip ~ source + (1 | site)
Data: cipresistant

Random effects:

Groups	Name	Variance	Std. Dev
site	(Intercept)	$5.885e^{-20}$	$2.426e^{-10}$

Number of obs: 36, groups: site, 12

Fixed effects:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-21.57	10335.23	-0.002	0.998
sourceStream	19.17	10335.23	0.002	0.999
sourceTreated	19.96	10335.23	0.002	0.998

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

	(Intr)	srcStr
sourceStrem	-1.000	
sourceTretd	-1.000	1.000

Ciprofloxacin resistant *E. coli* between and within regions using glmer.

Generalized linear mixed model fit by maximum likelihood (Adaptive Gauss-Hermite Quadrature, nAGQ = 0)
Family: binomial (logit)
Formula: cip ~ source * region + (1 | site)
Data: mixed

Random effects

Groups	Name	Variance	Std. Dev
site	(Intercept)	1.57e-15	3.963e-08

Number of obs: 36, groups: site, 12

Fixed effects:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	$-2.157e^{+01}$	$1.688e^{+04}$	-0.001	0.999
sourceStream	$-5.897e^{-06}$	$2.067e^{+04}$	0.000	1.000
sourceTreated	$2.087e^{+01}$	$1.688e^{+04}$	0.001	0.999
regionCant	$-6.007e^{-06}$	$2.387e^{+04}$	0.000	1.000
regionMarl	$-4.902e^{-06}$	$2.067e^{+04}$	0.000	1.000
sourceStream:regionCant	$5.897e^{-06}$	$3.157e^{+04}$	0.000	1.000
sourceTreated:regionCant	$-2.087e^{+01}$	$2.669e^{+04}$	-0.001	0.999
sourceStream:regionMarl	$2.087e^{+01}$	$2.387e^{+04}$	0.001	0.999
sourceTreated:regionMarl	$-2.087e^{+01}$	$2.669e^{+04}$	-0.001	0.999

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

	(Intr)	srcStr	srcTrt	rgnCnt	rgnMrl	srcS:C	srcT:C	srcS:M
sourceStrem	-0.816							
sourceTretd	-1.000	0.816						
regionCant	-0.707	0.577	0.707					
regionMarl	-0.816	0.667	0.816	0.577				
srcStrm:rgC	0.535	-0.655	-0.535	-0.756	-0.436			
srcTrtd:rgC	0.632	-0.516	-0.632	-0.894	-0.516	0.676		
srcStrm:rgM	0.707	-0.866	-0.707	-0.500	-0.866	0.567	0.447	
srcTrtd:rgM	0.632	-0.516	-0.632	-0.447	-0.775	0.338	0.400	0.671

A5 Comparison of chloramphenicol resistant *E. coli* populations

Chloramphenicol resistant *E. coli* in drinking water sources analysis using glmer.

Generalized linear mixed model fit by maximum likelihood (Adaptive Gauss-Hermite Quadrature, nAGQ = 0)
Family: binomial (logit)
Formula: chl ~ source + (1 | site)
Data: chlresistance

Random effects:

Groups	Name	Variance	Std. Dev
site	(Intercept)	10.31	3.212

Number of obs: 26, groups: site, 12

Fixed effects:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	$-2.257e^{+01}$	$1.704e^{+04}$	-0.001	0.999
sourceStream	$2.152e^{+01}$	$1.704e^{+04}$	0.001	0.999
sourceTreated	$-8.177e^{-07}$	$2.603e^{+04}$	0.000	1.000

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

	(Intr)	srcStr
sourceStrem	-1.000	
sourceTretd	-0.655	0.655

Chloramphenicol resistant *E. coli* between regions analysis using glmer.

Generalized linear mixed model fit by maximum likelihood (Adaptive Gauss-Hermite Quadrature, nAGQ = 0)
Family: binomial (logit)
Formula: chl ~ source * region + (1 | site)
Data: mixed

Random effects

Groups	Name	Variance	Std. Dev
site	(Intercept)	1.655	1.286

Number of obs: 36, groups: site, 12

Fixed effects:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	$-2.257e^{+01}$	$2.783e^{+04}$	-0.001	0.999
sourceStream	$2.337e^{+01}$	$2.783e^{+04}$	0.001	0.999
sourceTreated	$-2.668e^{-05}$	$3.935e^{+04}$	0.000	1.000
regionCant	$-2.668e^{-05}$	$3.935e^{+04}$	0.000	1.000
regionMarl	$-2.668e^{-05}$	$3.408e^{+04}$	0.000	1.000
sourceStream:regionCant	$-2.337e^{+01}$	$4.820e^{+04}$	0.000	1.000
sourceTreated:regionCant	$2.668e^{-05}$	$5.206e^{+04}$	0.000	1.000
sourceStream:regionMarl	$-2.337e^{+01}$	$4.400e^{+04}$	-0.001	1.000
sourceTreated:regionMarl	$2.668e^{-05}$	$5.206e^{+04}$	0.000	1.000

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

	(Intr)	srcStr	srcTrt	rgnCnt	rgnMrl	srcS:C	srcT:C	srcS:M
sourceStrem	-1.000							
sourceTretd	-0.707	0.707						
regionCant	-0.707	0.707	0.500					
regionMarl	-0.816	0.816	0.577	0.577				
srcStrm:rgC	0.577	-0.577	-0.408	-0.816	-0.471			
srcTrtd:rgC	0.535	-0.535	-0.756	-0.756	-0.436	0.617		
srcStrm:rgM	0.632	-0.632	-0.447	-0.447	-0.775	0.365	0.338	
srcTrtd:rgM	0.535	-0.535	-0.756	-0.378	-0.655	0.309	0.571	0.507

A6 Okains Bay seasonal differences on *E. coli* counts

The effect of season on *E. coli* counts using glmer.

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)
Family: poisson (log)
Formula: ecoli ~ season + (1 | site) + (1 | obs)
Data: ok2

Random effects:

Groups	Name	Variance	Std. Dev
obs	(Intercept)	1.327	1.152
site	(Intercept)	1.126	1.061

Number of obs: 67, groups: obs, 67; site, 6

Fixed effects:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	4.0875	0.5237	7.805	$5.95e^{-15}$ ***
seasonSpring	-0.2003	0.4215	-0.475	0.63470
seasonAutumn	-1.1661	0.4123	-2.828	0.00468 **
seasonSummer	0.6821	0.4064	1.678	0.09330

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

	(Intr)	srcStr	srcTrt
seasonSpring	-0.375		
seasonAutumn	-0.403	0.478	
seasonSummer	-0.408	0.485	0.526

Emmeans post hoc analysis:

Contrasts	Estimate	Std. Error	z ratio	p value
Winter - Spring	0.200	0.421	0.475	0.9646
Winter - Autumn	1.166	0.412	2.828	0.0242
Winter - Summer	-0.682	0.406	-1.678	0.3352
Spring - Autumn	0.966	0.426	2.266	0.1061
Spring - Summer	-0.882	0.420	-2.099	0.1535
Autumn - Summer	-1.848	0.399	-4.635	<.0001

A7 Okains Bay seasonal differences on ampicillin resistant *E. coli* counts

The effect of season on ampicillin resistant *E. coli* counts using glmer.

Generalized linear mixed model fit by maximum likelihood (Adaptive Gauss-Hermite Quadrature, nAGQ = 0)
Family: poisson (log)
Formula: amp ~ season + (1 | site) + (1 | obs)
Data: ok2

Random effects:

Groups	Name	Variance	Std. Dev
obs	(Intercept)	0.4802	0.6930
site	(Intercept)	0.8012	0.8951

Number of obs: 67, groups: obs, 67; site, 6

Fixed effects:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	3.6466	0.4087	8.923	$< 2e^{-16}$ ***
seasonSpring	-0.3679	0.2638	-1.394	0.163
seasonAutumn	-25.0428	5488.8596	-0.005	0.996
seasonSummer	-1.5973	0.2698	-5.920	$3.22e^{-09}$ ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

	(Intr)	srcStr	srcTrt
seasonSpring	-0.289		
seasonAutumn	0.000	0.000	
seasonSummer	-0.309	0.435	0.000

Emmeans post hoc analysis:

Contrasts	Estimate	Std. Error	z ratio	p value
Winter - Spring	0.368	0.264	1.394	0.5029
Winter - Autumn	25.043	5488.860	0.005	1.0000
Winter - Summer	1.597	0.270	5.920	<.0001
Spring - Autumn	24.675	5488.860	0.004	1.0000
Spring - Summer	1.229	0.284	4.335	0.0001
Autumn - Summer	-23.446	5488.860	-0.004	1.0000

A8 Okains Bay *E. coli* count differences across sites: Winter

The variation of *E. coli* counts across the six sites in winter using glm. Bridge (B), Estuary (E), Camp Kitchen (K), Schoolhouse (S), Tributary (T), Upstream (U).

glm(formula = ecoli ~ site, family = poisson(link = "log"), data = winter)

Coefficients:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	4.05468	0.07603	53.331	$< 2e^{-16}$ ***
siteE	-0.55817	0.12602	-4.429	$9.46e^{-06}$ ***
siteK	-0.54812	0.12562	-4.363	$1.28e^{-05}$ ***
siteS	0.06706	0.10576	0.634	0.5260
siteT	0.33977	0.13463	2.524	0.0116 *
siteU	0.47073	0.09690	4.858	$1.19e^{-06}$ ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Emmeans post hoc analysis: only p values less than 0.05 shown.

Contrasts	Estimate	Std. Error	z ratio	p value
B - E	0.5582	0.1260	4.429	0.0001
B - K	0.5481	0.1256	4.363	0.0002
B - U	-0.4707	0.0969	-4.858	<.0001
E - S	-0.6252	0.1245	-5.021	<.0001
E - T	-0.8979	0.1498	-5.993	<.0001
E - U	-1.0289	0.1171	-8.787	<.0001
K - S	-0.6152	0.1241	-4.956	<.0001
K - T	-0.8879	0.1495	-5.940	<.0001
K - U	-1.0188	0.1167	-8.733	<.0001
S - U	-0.4037	0.0950	-4.251	0.0003

The variation in ampicillin resistant *E. coli* counts across the six sites analysis.

glm(formula = amp ~ site, family = poisson(link = "log"), data = winter)

Coefficients:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	4.14841	0.07255	57.182	$< 2e^{-16}$ ***
siteE	-1.02752	0.14131	-7.271	$3.56e^{-13}$ ***
siteK	-1.16949	0.14904	-7.847	$4.26e^{-15}$ ***
siteS	-0.29826	0.11115	-2.683	0.00729 **
siteT	-0.19717	0.15651	-1.260	0.20774
siteU	-0.22314	0.10882	-2.051	0.04031 *

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Emmeans post hoc analysis: only p values less than 0.05 shown.

Contrasts	Estimate	Std. Error	z ratio	p value
B - E	1.0275	0.141	7.271	<.0001
B - K	1.1695	0.149	7.847	<.0001
E - S	-0.7293	0.148	-4.939	<.0001
E - T	-0.8303	0.184	-4.507	0.0001
E - U	-0.8044	0.146	-5.513	<.0001
K - S	-0.8712	0.155	-5.619	<.0001
K - T	-0.9723	0.190	-5.112	<.0001
K - U	-0.9463	0.153	-6.170	<.0001

A9 Okains Bay *E. coli* count differences across sites: Spring

The variation of *E. coli* counts across the six sites in spring using glm. Bridge (B), Estuary (E), Camp Kitchen (K), Schoolhouse (S), Tributary (T), Upstream (U).

glm(formula = ecoli ~ site, family = poisson(link = "log"), data = spring)

Coefficients:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	4.53618	0.05976	75.905	$< 2e^{-16}$ ***
siteE	-1.31730	0.13002	-10.132	$< 2e^{-16}$ ***
siteK	-3.43757	0.33865	-10.151	$< 2e^{-16}$ ***
siteS	0.57179	0.07475	7.649	$2.02e^{-14}$ ***
siteU	-0.43629	0.09537	-4.575	$4.77e^{-06}$ ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Emmeans post hoc analysis: only p values less than 0.05 shown.

Contrasts	Estimate	Std. Error	z ratio	p value
B - E	1.317	0.1300	10.132	<.0001
B – K	3.438	0.3386	-7.649	<.0001
B – U	0.436	0.0954	4.575	<.0001
E – K	2.120	0.3528	6.010	<.0001
E – S	-1.889	0.1239	-15.248	<.0001
E – U	-0.881	0.1373	-6.415	<.0001
K – S	-4.009	0.3363	-11.920	<.0001
K – U	-3.001	0.3415	-8.788	<.0001
S – U	1.008	0.0868	11.609	<.0001

Variation of ampicillin resistant *E. coli* counts across the six sites in spring using glm.

glm(formula = amp ~ site, family = poisson(link = "log"), data = spring)

Coefficients:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	4.20966	0.07036	59.830	$< 2e^{-16}$ ***
siteE	-1.24782	0.14897	-8.376	$< 2e^{-16}$ ***
siteK	-3.92197	0.50493	-7.767	$8.01e^{-15}$ ***
siteS	-0.12648	0.10280	-1.230	0.219
siteU	-0.67354	0.12108	-5.563	$2.65e^{-08}$ ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Emmeans post hoc analysis: only p values less than 0.05 shown.

Contrasts	Estimate	Std. Error	z ratio	p value
B – E	1.248	0.149	8.376	<.0001
B – K	3.922	0.505	7.767	<.0001
B – U	0.674	0.121	5.563	<.0001
E – K	2.674	0.517	5.173	<.0001
E – S	-1.121	0.151	-7.417	<.0001
E – U	-0.574	0.164	-3.498	0.0043
K – S	-3.795	0.506	-7.507	<.0001
K – U	-3.248	0.510	-6.374	<.0001
S – U	0.547	0.124	4.419	0.0001

Variation of chloramphenicol resistant *E. coli* counts across the six sites in spring using glm.

glm(formula = chl ~ site, family = poisson(link = "log"), data = spring)

Coefficients:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	0.2877	0.5000	0.575	0.565
siteE	-20.5903	8973.0992	-0.002	0.998
siteK	-20.5903	8973.0992	-0.002	0.998
siteS	2.4423	0.5213	4.685	$2.8e^{-06}$ ***
siteU	0.5596	0.6268	0.893	0.372

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Emmeans post hoc analysis: only p values less than 0.05 shown.

Contrasts	Estimate	Std. Error	z ratio	p value
B – S	-2.44	$5.21e^{-01}$	-4.685	<.0001
S – U	1.88	$4.06e^{-01}$	4.641	<.0001

A10 Okains Bay *E. coli* count differences across sites: Autumn

Variation of *E. coli* counts across the six sites in autumn using glm. (B), Estuary (E), Camp Kitchen (K), Schoolhouse (S), Tributary (T), Upstream (U).

glm(formula = ecoli ~ site, family = poisson(link = "log"), data = aut)

Coefficients:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	4.65713	0.05625	82.787	< 2e^{-16} ***
siteE	-3.96398	0.41211	-9.619	< 2e^{-16} ***
siteK	-1.36129	0.12454	-10.931	< 2e^{-16} ***
siteT	-0.65588	0.09624	-6.815	9.43e^{-12} ***
siteU	-0.14261	0.08255	-1.728	0.0841

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Emmeans post hoc analysis: only p values less than 0.05 shown.

Contrasts	Estimate	Std. Error	z ratio	p value
B – E	3.964	0.4121	9.619	<.0001
B – K	1.361	0.1245	10.931	<.0001
B – T	0.656	0.0962	6.815	<.0001
E – K	-2.603	0.4231	-6.151	<.0001
E – T	-3.308	0.4156	-7.959	<.0001
E - U	-3.821	0.4127	-9.260	<.0001
K – T	-0.705	0.1358	-5.194	<.0001
K – U	-1.219	0.1265	-9.636	<.0001
T – U	-0.513	0.0987	-5.199	<.0001

Variation of ciprofloxacin resistant *E. coli* counts across the six sites in autumn using glm.

glm(formula = cip ~ site, family = poisson(link = "log"), data = aut)

Coefficients:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	3.71357	0.09017	41.186	< 2e^{-16} ***
siteE	-4.81218	1.00403	-4.793	1.64e^{-06} ***
siteK	-3.02042	0.41809	-7.224	5.03e^{-13} ***
siteT	-0.62253	0.15258	-4.080	4.50e^{-05} ***
siteU	0.11507	0.12400	0.928	0.353

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Emmeans post hoc analysis: only p values less than 0.05 shown.

Contrasts	Estimate	Std. Error	z ratio	p value
B – E	4.812	1.004	4.793	<.0001
B – K	3.020	0.418	7.224	<.0001
B – T	0.623	0.153	4.080	0.0004
E – T	-4.190	1.008	-4.158	0.0003
E – U	-4.927	1.004	-4.910	<.0001
K – T	-2.398	0.426	-5.624	<.0001
K – U	-3.135	0.417	-7.519	<.0001
T – U	-0.738	0.150	-4.929	<.0001

A11 Okains Bay *E. coli* count differences across sites: Summer

Variation of *E. coli* counts across the six sites in summer using glm. (B), Estuary (E), Camp Kitchen (K), Schoolhouse (S), Tributary (T), Upstream (U).

glm(formula = ecoli ~ site, family = poisson(link = "log"), data = summer)

Coefficients:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	5.99479	0.02882	208.012	< 2e^{-16} ***
siteE	-7.09340	1.00039	-7.091	1.33e^{-12} ***
siteK	-1.45862	0.06635	-21.984	< 2e^{-16} ***
siteS	-0.19772	0.04293	-4.606	4.10e^{-06} ***
siteT	0.91296	0.03412	26.761	< 2e^{-16} ***
siteU	-1.06513	0.05692	-18.712	< 2e^{-16} ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Emmeans post hoc analysis: only p values less than 0.05 shown.

Contrasts	Estimate	Std. Error	z ratio	p value
B – E	7.093	1.0004	7.091	<.0001
B – K	1.459	0.0663	21.984	<.0001
B – S	0.198	0.0429	4.606	0.0001
B – T	-0.913	0.0341	-26.761	<.0001
B – U	1.065	0.0569	18.712	<.0001
E – K	-5.635	1.0018	-5.625	<.0001
E – S	-6.896	1.0005	-6.892	<.0001
E – T	-8.006	1.0001	-8.005	<.0001
E – U	-6.028	1.0012	-6.021	<.0001
K – S	-1.261	0.0677	-18.624	<.0001
K – T	-2.372	0.0625	-37.952	<.0001
K – U	-0.393	0.0773	-5.088	<.0001
S – T	-1.111	0.0367	-30.280	<.0001
S – U	0.867	0.0585	14.828	<.0001
T – U	1.978	0.0524	37.769	<.0001

The variation of ampicillin resistant *E. coli* counts across the six sites in summer using glm.

glm(formula = amp ~ site, family = poisson(link = "log"), data = summer)

Coefficients:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	3.1918	0.1170	27.271	$< 2e^{-16}$ ***
siteE	-4.2905	1.0068	-4.261	$2.03e^{-05}$ ***
siteK	-2.4987	0.4247	-5.884	$4.02e^{-09}$ ***
siteS	0.7068	0.1430	4.942	$7.75e^{-07}$ ***
siteT	-0.1633	0.1727	-0.946	0.344
siteU	-2.9042	0.5135	-5.655	$1.55e^{-08}$ ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Emmeans post hoc analysis: only p values less than 0.05 shown.

Contrasts	Estimate	Std. Error	z ratio	p value
B – E	4.290	1.007	4.261	0.0003
B – K	2.499	0.425	5.884	<.0001
B – S	-0.707	0.143	5.884	<.0001
B – U	2.904	0.514	5.655	<.0001
E – S	-4.997	1.003	-4.981	<.0001
E – T	-4.127	1.008	-4.094	0.0006
K – S	-3.205	0.416	-7.697	<.0001
K – T	-2.335	0.428	-5.462	<.0001
S – T	0.870	0.151	5.751	<.0001
S – U	3.611	0.507	7.126	<.0001
T – U	2.741	0.516	5.313	<.0001

A12 Comparison of multidrug resistant *E. coli*

The comparison of multidrug resistant *E. coli* in drinking water sources using glmer.

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation) [glmerMod]
Family: poisson (log)
Formula: total_mdr ~ source + (1 | site) + (1 | mdr$obs_effect)
Data: mdr

Random effects:

Groups	Name	Variance	Std. Dev
mdr$obs_effect	(Intercept)	0.268	0.5177
site	(Intercept)	5.491	2.3432

Number of obs: 36, groups: mdr$obs_effect, 36; site, 12

Fixed effects:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	0.8971	1.3593	0.660	0.5092
sourceStream	1.3552	1.8035	0.751	0.4524
sourceTreated	-3.9775	1.9836	-2.005	0.0449 *

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

	(Intr)	srcStr
sourceStrem	-0.753	
sourceTretd	-0.623	0.470

Emmeans post hoc analysis:

Contrasts	Estimate	Std. Error	z ratio	p value
River – Stream	-1.36	1.80	-0.751	0.7327
River – Treated	3.98	1.98	2.005	0.1109
Stream – Treated	5.33	1.96	2.726	0.0176

The comparison of multidrug resistant *E. coli* between and within regions using glmer.

Generalized linear mixed model fit by maximum likelihood (Adaptive Gauss-Hermite Quadrature, nAGQ = 0) [glmerMod]
Family: poisson (log)
Formula: total_mdr ~ source * region + (1 | site) + (1 | mdr$obs_effect)
Data: mdr

Random effects

Groups	Name	Variance	Std. Dev
mdr$obs_effect	(Intercept)	0.2638	0.5136
site	(Intercept)	1.6710	1.2927

Number of obs: 36, groups: mdr$obs_effect, 36; site, 12

Fixed effects:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	4.251	1.329	3.200	0.00137 **
sourceStream	-2.483	1.635	-1.519	0.12881
sourceTreated	-5.357	2.127	-2.518	0.01180 *
regionCant	-23.554	5442.434	-0.004	0.99655
regionMarl	-2.521	1.671	-1.509	0.13140
sourceStream:regionCant	26.256	5442.434	0.005	0.99615
sourceTreated:regionCant	22.750	5442.434	0.004	0.99666
sourceStream:regionMarl	1.955	2.358	0.829	0.40700
sourceTreated:regionMarl	-15.676	5442.460	-0.003	0.99770

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

	(Intr)	srcStr	srcTrt	rgnCnt	rgnMrl	srcS:C	srcT:C	srcS:M
sourceStrem	-0.813							
sourceTretd	-0.625	0.507						
regionCant	0.000	0.000	0.000					
regionMarl	-0.795	0.646	0.496	0.000				
srcStrm:rgC	0.000	0.000	0.000	-1.000	0.000			
srcTrtd:rgC	0.000	0.000	0.000	-1.000	0.000	1.000		
srcStrm:rgM	0.563	-0.693	-0.352	0.000	-0.709	0.000	0.000	
srcTrtd:rgM	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Emmeans post hoc analysis: only p values less than 0.05 shown.

Contrasts	Estimate	Std. Error	z ratio	p value
River Banks - Treated Cant	$6.16e^{+00}$	1.94	3.181	0.0393
Stream Cant - Treated Cant	$6.38e^{+00}$	1.94	3.295	0.0274

A13 Okains Bay seasonal differences on multidrug resistant *E. coli*

The comparison of multidrug resistant *E. coli* across seasons in Okains Bay. (B), Estuary (E), Camp Kitchen (K), Schoolhouse (S), Tributary (T), Upstream (U).

Generalized linear mixed model fit by maximum likelihood (Laplace Approximation) [glmerMod]
Family: poisson (log)
Formula: mdr ~ season + (1 | strain)
Data: obmdr1

Random effects:

Groups	Name	Variance	Std. Dev
strain	(Intercept)	0.6625	0.8139

Number of obs: 24, groups: strain, 24

Fixed effects:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-0.1704	0.5968	-0.286	0.775
seasonSpring	0.5246	0.7210	0.728	0.467
seasonSummer	0.2849	0.7329	0.389	0.698
seasonWinter	0.7546	0.7066	1.068	0.286

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

	(Intr)	ssnSpr	ssnSmm
seasonSpring	-0.746		
seasonSummer	-0.714	0.563	
seasonWinter	-0.759	0.592	0.574

Emmeans post hoc analysis:

Contrasts	Estimate	Std. Error	z ratio	p value
Autumn - Spring	-0.525	0.721	-0.728	0.8860
Autumn - Summer	-0.285	0.733	-0.389	0.9801
Autumn - Winter	-0.755	0.707	-1.068	0.7091
Spring - Summer	0.240	0.680	0.353	0.9849
Spring - Winter	-0.230	0.645	-0.357	0.9845
Spring - Winter	-0.470	0.665	-0.706	0.8946

The comparison of multidrug resistant *E. coli* across sites in Okains Bay.

Generalized linear mixed model fit by maximum likelihood (Adaptive Gauss-Hermite Quadrature, nAGQ = 0) [glmerMod]
Family: poisson (log)
Formula: mdr ~ site + (1 | strain)
Data: obmdr

Random effects:

Groups	Name	Variance	Std. Dev
strain	(Intercept)	$5.534e^{-18}$	$2.352e^{-09}$

Number of obs: 27, groups: strain, 27

Fixed effects:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	$1.253e^{+00}$	$2.673e^{-01}$	4.687	$2.77e^{-06}$ ***
siteE	$-8.473e^{-01}$	$4.879e^{-01}$	-1.737	0.0825
siteK	$-1.540e^{+00}$	$6.360e^{-01}$	-2.422	0.0154 *
siteResidential	$1.362e^{+00}$	$3.095e^{-01}$	4.401	$1.08e^{-05}$ ***
siteS	$-1.253e^{+00}$	$5.669e^{-01}$	-2.210	0.0271 *
siteT	$-2.639e^{+00}$	$1.035e^{+00}$	-2.550	0.0108 *
siteU	$-2.763e^{-12}$	$3.780e^{-01}$	0.000	1.0000

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

	(Intr)	siteE	siteK	stRsdn	siteS	siteT
siteE	-0.548					
siteK	-0.420	0.230				
siteResdntl	-0.863	0.473	0.363			
siteS	-0.471	0.258	0.198	0.407		
siteT	-0.258	0.141	0.108	0.223	0.122	
siteU	-0.707	0.387	0.297	0.611	0.333	0.183

Emmeans post hoc analysis: only p values less than 0.05 shown.

Contrasts	Estimate	Std. Error	z ratio	p value
B – Residential	-1.362	0.310	-4.401	0.0002
E – Residential	-2.209	0.437	-5.055	<.0001
K – Residential	-2.903	0.598	-4.855	<.0001
Residential – S	2.615	0.524	4.992	<.0001
Residential – T	4.001	1.012	3.953	0.0015
Residential – U	1.362	0.310	4.401	0.0002

Appendix B: MDR *E. coli* isolates from the antibiotic susceptibility assay

Phenotypic confirmation of antibiotic susceptibility profiles of MDR isolates including the ESBL screening information. MDR phenotype includes resistance to Ctx, Cla, Ctx/Cla and Caz/Cla. 0 = Susceptible; 1 = Intermediate Resistance; 2 = Resistant; - = Not tested. Please see Abbreviations list at the beginning of this thesis for abbreviation definitions.

Strain	Source	Location	Amp	Chl	Rif	Tet	Kan	Tmp	Gen	Cip	Ctx	Caz	Ctx / Cla	Caz / Cla
OB2A4	Other	BP	2	1	0	0	0	0	2	2	-	-	-	-
OB18	Other	BP	2	0	0	0	0	0	2	0	2	0	2	0
OB21	Other	BP	2	0	0	0	0	0	2	2	-	-	-	-
2OB1G9	Other	BP	2	0	2	0	2	0	2	0	-	-	-	-
2OB1G10	Other	BP	2	0	0	0	2	0	1	2	-	-	-	-
3OB1A3	Other	BP	2	0	0	0	0	2	2	0	-	-	-	-
3OB1A10	Other	BP	2	0	0	0	0	2	2	2	-	-	-	-
3OB1A15	Other	BP	2	0	0	0	0	2	2	0	-	-	-	-
3OA18	Other	BP	2	1	0	0	0	0	2	2	0	0	0	0
3OB1A11	Other	BP	2	0	0	0	0	2	2	2	2	0	2	0
3OB1A1001	Other	BP	2	0	0	0	0	2	2	0	2	0	2	0
3OB1A1003	Other	BP	2	0	0	0	2	0	2	0	-	-	-	-
3OB2A12	Other	BP	2	0	0	0	2	0	2	0	-	-	-	-
3OB2A5	Other	BP	2	0	0	0	2	2	2	0	-	-	-	-
3OB2A3	Other	BP	2	0	0	0	0	2	1	2	-	-	-	-
3OG10	Other	BP	2	1	0	0	0	0	2	2	2	0	0	0
SOU1	Other	BP	0	0	2	0	1	0	2	0	2	0	2	0
AUS1C	Stream	BP	2	0	0	0	0	0	1	0	2	0	2	0
AUS3A	Stream	BP	2	0	0	0	0	0	2	0	2	0	2	0
AB1A	Stream	BP	1	0	0	1	2	0	2	0	2	0	2	0
AB2A	Stream	BP	2	1	0	1	2	0	2	0	2	0	2	0
AB2C	Stream	BP	1	1	0	1	0	0	2	0	2	0	2	0
AS1A	Stream	BP	1	0	0	0	1	0	2	0	2	0	2	0
AS3A	Stream	BP	1	1	0	0	0	0	2	0	2	0	2	0
AE1A	Stream	BP	1	0	0	0	2	0	2	0	2	0	2	0
AE2G	Stream	BP	1	0	0	0	1	0	2	0	2	0	2	0
ATG	Stream	BP	1	0	0	0	1	0	2	0	2	0	2	0
AE1J	Stream	BP	2	1	0	0	0	0	2	0	2	0	2	0
U2CHL	Stream	BP	2	0	2	1	2	0	2	0	0	0	0	0
S2E	Stream	BP	2	0	0	0	2	0	2	0	2	0	2	0
S1A	Stream	BP	2	0	0	0	1	0	1	0	2	2	2	0
U2CHL	Stream	BP	0	0	0	2	0	0	2	0	2	0	2	0
U32	Stream	BP	0	0	0	0	0	0	0	2	2	0	2	0
U1C5	Stream	BP	2	1	0	0	0	0	0	2	2	2	0	2
U14	Stream	BP	0	0	0	1	0	0	0	2	2	0	2	0
TRIB3C4	Stream	BP	0	0	0	1	0	0	1	2	2	2	2	2
TRIB22	Stream	BP	1	1	0	1	0	0	1	2	2	0	2	0
TRIB12	Stream	BP	1	1	0	1	0	0	1	2	2	0	2	0
PC3C5	Stream	BP	0	0	0	1	0	0	1	2	2	0	2	0
PC2C1	Stream	BP	2	2	0	1	0	0	0	2	2	0	2	0
PC2AMP	Stream	BP	2	0	0	2	0	0	1	2	2	0	2	0

PC1C5	Stream	BP	1	2	0	1	0	0	1	2	2	0	2	0
PC1C1	Stream	BP	2	2	0	2	0	0	0	2	0	2	0	2
OVU35	Other	BP	1	0	2	1	0	2	1	2	-	-	-	-
OVU2C5	Other	BP	0	0	0	1	0	0	0	2	2	0	2	0
OVU2C1	Other	BP	0	0	0	1	0	0	0	2	2	0	2	0
OVD31	Other	BP	1	1	0	1	0	0	1	2	2	0	2	0
OVD13	Other	BP	1	0	0	0	0	0	1	2	0	2	0	2
OVD12	Other	BP	1	1	0	1	0	0	1	2	2	2	0	0
OVD11	Other	BP	0	1	0	1	0	0	1	2	2	0	2	0
OKANACHL	Other	BP	2	2	0	2	0	2	1	2	0	0	0	0
OKANAAMP	Other	BP	2	2	0	2	0	2	1	2	0	0	0	0
OKANA4	Other	BP	1	0	0	0	0	0	0	2	0	2	0	2
LRC1CIP	Treated	BP	0	0	0	0	1	2	1	2	2	2	2	2
K25	Stream	BP	1	1	0	1	0	0	0	2	2	0	2	0
E21	Stream	BP	0	1	0	0	0	0	1	2	2	0	2	0
E1CIP	Stream	BP	1	1	0	0	0	0	1	2	2	0	2	0
B3C3	Stream	BP	0	0	2	0	0	0	1	2	0	2	0	2
B1C1	Stream	BP	0	1	0	0	0	0	1	2	0	2	0	2
AU12	Stream	BP	2	0	0	2	0	0	2	0	-	-	-	-
OM3A	Treated	C	2	0	0	2	0	0	2	0	0	2	0	2
SF11	Stream	C	2	0	0	2	0	0	2	2	-	-	-	-
SF12	Stream	C	2	0	0	2	0	0	2	2	0	0	0	0
SF13	Stream	C	1	0	0	2	0	0	2	2	0	0	0	0
SF15	Stream	C	1	0	0	2	0	0	2	2	0	0	0	0
SF17	Stream	C	2	0	0	2	0	0	2	2	-	-	-	-
SF19	Stream	C	2	0	0	2	0	0	2	2	-	-	-	-
SF10	Stream	C	2	0	0	2	0	0	0	2	-	-	-	-
WHI22A	Stream	MS	2	0	2	0	0	0	0	2	0	0	0	0
WHI22C	Stream	MS	2	0	2	0	0	0	0	2	0	2	0	0
WHI23A	Stream	MS	2	0	2	0	0	0	0	2	0	0	0	0
WHI24A	Stream	MS	2	0	2	2	0	0	0	2	0	0	0	0
WHA11	Other	MS	2	2	0	2	0	0	2	0	-	-	-	-
WHA12	Other	MS	2	2	0	2	0	0	2	0	-	-	-	-
WHA13	Other	MS	2	2	0	2	2	0	2	0	-	-	-	-
WHA14	Other	MS	2	0	0	2	1	0	2	0	-	-	-	-
WHA15	Other	MS	2	1	0	2	0	0	2	0	-	-	-	-
WHA16	Other	MS	2	2	0	2	0	0	2	0	-	-	-	-
WHA17	Other	MS	2	1	0	2	0	0	2	0	-	-	-	-
WHA18	Other	MS	2	0	0	2	0	0	2	0	-	-	-	-
WHA19	Other	MS	2	0	0	2	0	0	2	0	-	-	-	-
WHA10	Other	MS	2	0	0	2	0	0	2	0	-	-	-	-
WHA24	Other	MS	0	2	0	0	0	0	2	2	0	0	0	0
2WHA3A6	Other	MS	2	0	0	1	0	0	2	2	2	2	2	0
WB3A6	Other	MS	2	0	0	1	0	0	2	2	2	2	2	0
3WHA32	Other	MS	1	1	0	1	2	0	2	0	2	0	2	0
WH24	Stream	MS	1	0	0	0	0	0	1	2	2	2	2	0

Appendix C: ESBL- and AmpC-producing isolates

Isolates with phenotypic confirmation of ESBL- or AmpC-production. *ESBL-producer; S = Susceptible; R = Resistant. Please see Abbreviations list at the beginning of this thesis for abbreviation definitions.

***E. coli* strain**	**Amp^R**	**Cip^R**	**CTX**	**CAZ**	**CTX / CLA**	**CAZ / CLA**	**Isolated from**	**Location**
2WHA26	R	S	R	S	R	R	Other	MS
2WHA37	R	S	R	S	R	R	Other	MS
3OB1A14	R	S	R	S	R	R	Other	BP
OB2	R	R	R	S	R	R	Other	BP
OB2A11	R	R	R	S	R	R	Other	BP
OB2A9	R	R	R	S	R	R	Other	BP
OKANACHL	R	R	R	S	R	R	Other	BP
OVD11*	S	R	R	S	S	S	Other	BP
OVD12*	S	R	R	S	S	S	Other	BP
OVD13*	S	R	S	R	S	S	Other	BP
OVD1C1*	S	R	R	R	S	S	Other	BP
OVD21	S	R	R	S	R	R	Other	BP
OVD31	S	R	S	R	R	R	Other	BP
OVU24	S	R	R	R	R	R	Other	BP
OVU2C5	S	R	R	S	R	R	Other	BP
WHA24	S	R	R	R	R	R	Other	MS
1B4A	R	R	R	S	R	R	Stream	BP
3B4A	R	R	R	S	R	R	Stream	BP
AB2A	R	S	R	R	R	R	Stream	BP
AS2A	R	S	R	S	R	R	Stream	BP
AUS1D	R	S	R	S	R	R	Stream	BP
AUS2B	S	S	R	S	R	R	Stream	BP
B1C1*	S	R	R	R	R	R	Stream	BP
B2CHL	S	R	S	R	R	R	Stream	BP
E21	S	R	R	S	R	R	Stream	BP
E2B*	R	R	R	S	S	S	Stream	BP
E31	S	R	R	S	R	R	Stream	BP
K13	S	R	R	R	R	R	Stream	BP
K25	S	R	R	S	R	R	Stream	BP
K3C3	S	R	R	S	R	R	Stream	BP
PC1C5	S	R	R	S	R	R	Stream	BP
PC21	S	R	R	S	R	R	Stream	BP
PC2AMP	R	R	S	R	R	R	Stream	BP
PC2C1	R	R	R	R	R	R	Stream	BP
SF1*	R	R	S	R	S	S	Stream	C
WHI23A	R	R	R	S	R	R	Stream	MS
WHI24B	R	S	R	S	R	R	Stream	MS
LRC1CIP	S	R	R	S	R	R	Treated	BP

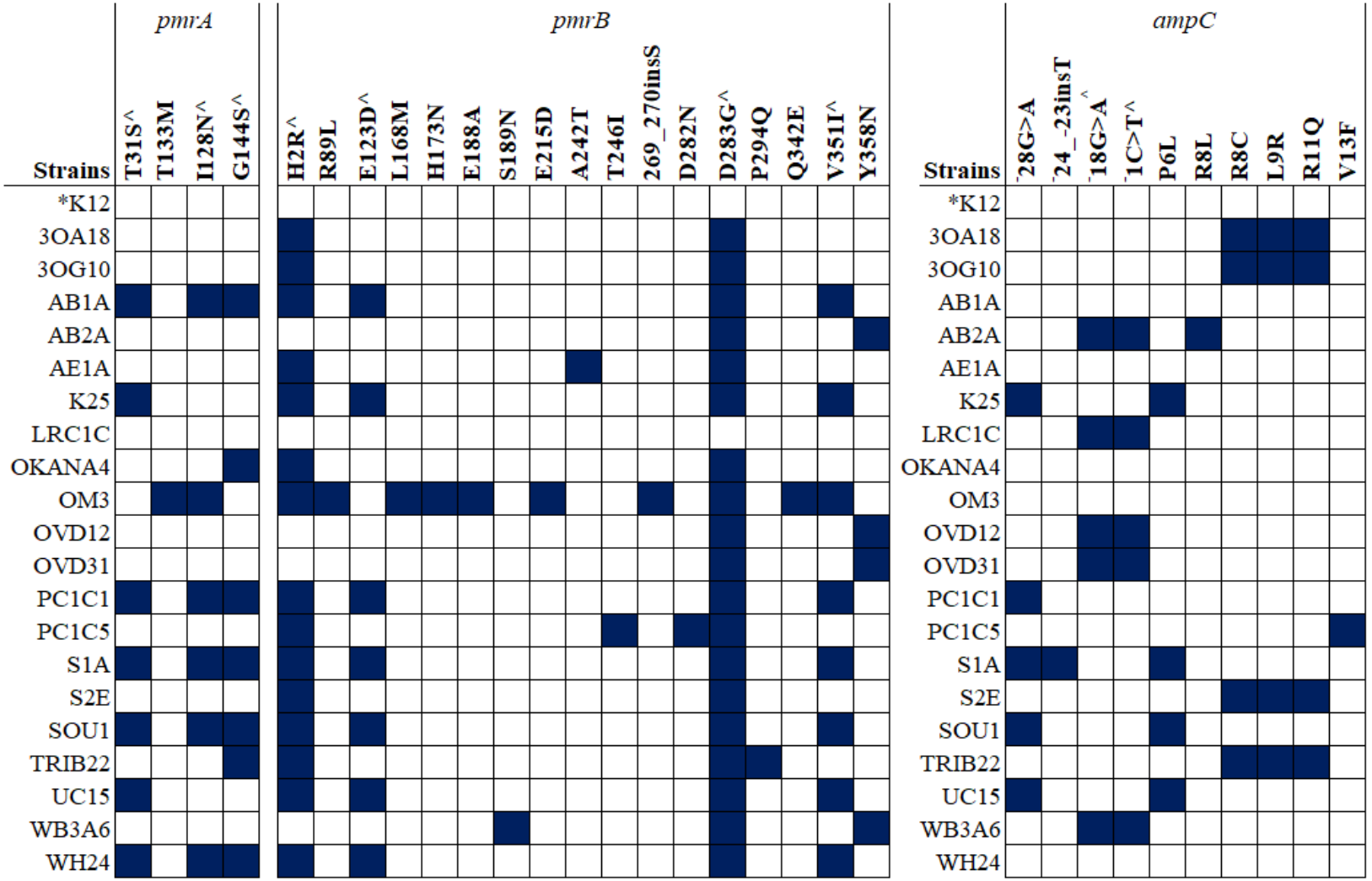
Appendix D: Classification of the *sequences* E. coli isolates

Results from SerotypeFinder 2.0 v2.0.1, Pathogen Finder v1.1 and MLST 2.0 v2.0.4. ST = sequence type; *Novel ST.

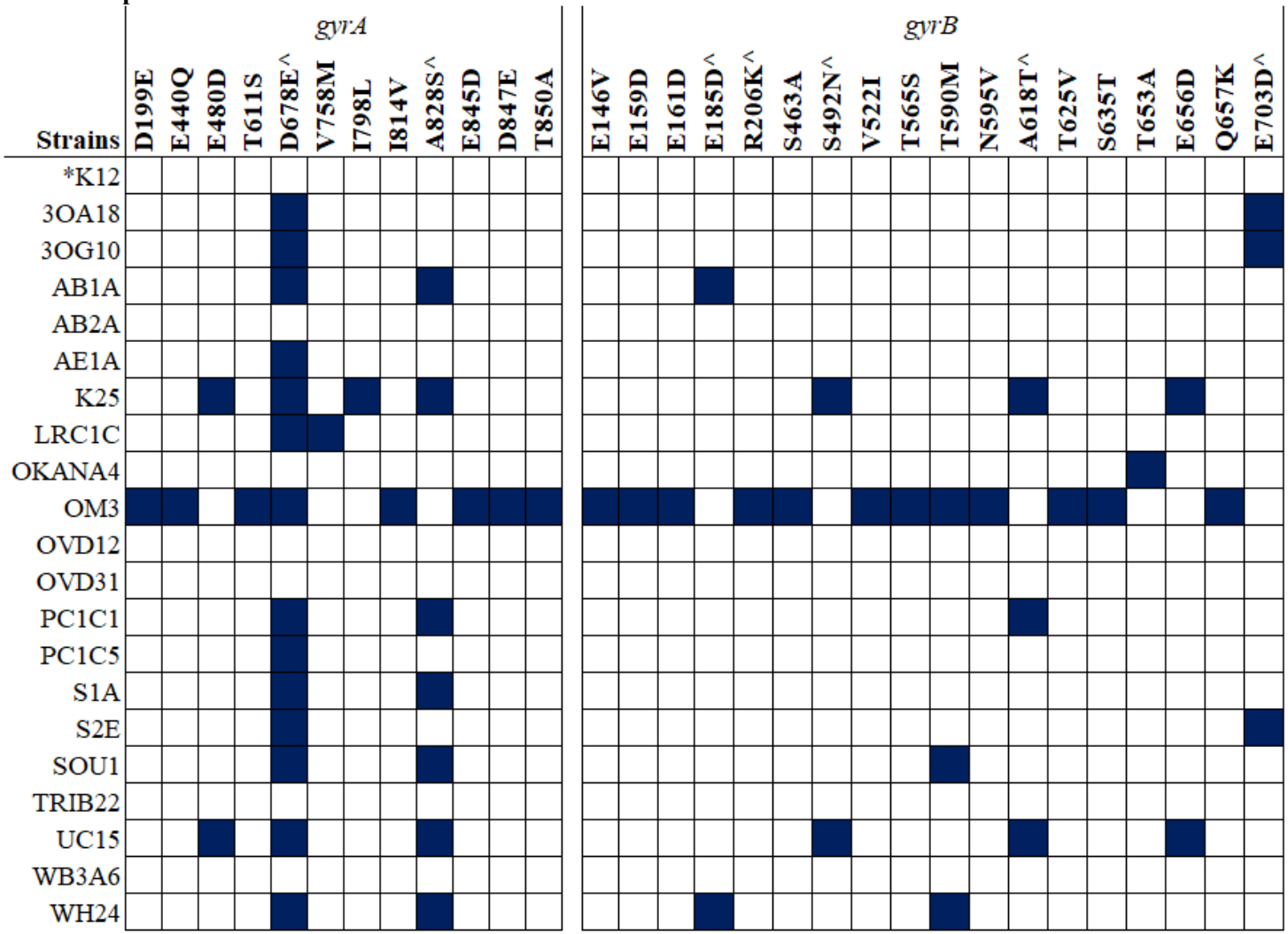
***E. coli* strain**	**Serotype**	**Predicted Human Pathogen**	**MLST E. coli #1**	**MLST E. coli #2**
3OBA18	O27:H46	0.941	ST Unknown	Nearest ST273*
3OG10	O27:H46	0.941	ST Unknown	ST Unknown*
AB1A	O4:H5	0.923	ST1	ST Unknown
AB2A	O39:H28	0.941	ST6096	ST726
AE1A		0.937	ST8979	ST Unknown*
K25	O8:H10	0.93	ST681	ST304
LRC1C	O98:H41	0.937	ST1087	ST Unknown*
OKANA4	O17/O77:H34	0.943	ST Unknown*	ST816
OM3	O103:H56	0.891	ST5260	Nearest ST989*
OVD12	O156:H7	0.945	ST2539	ST323
OVD31	O156:H7	0.944	ST2539	ST323
PC1C1	H10^	0.941	ST1865*	Nearest ST803
PC1C5	O166:H15	0.935	ST349	ST678*
S1A	O170:H5	0.937	ST3307	Nearest ST324
S2E	O27:H46	0.941	ST Unknown	ST Unknown*
SOU1	O87:H6	0.939	ST3303	ST725
TRIB22	H9^	0.939	ST Unknown*	ST Unknown*
UC15	O8:H10	0.933	ST681	ST304
WB3A6	O88:H8	0.937	ST446	ST397
WH24	O132:H34	0.938	ST582	ST510

Appendix E: Point mutations with unknown phenotypes

E1: Point mutations in *pmrA*, *pmrB* and *ampC* that were identified in PointFinder. These mutations were associated with resistance; however, the phenotype was unknown. ^indicates point mutations that have been identified in the literature before.



E2: Point mutations *gyrA* and *gyrB* that were identified in PointFinder. These mutations were associated with resistance; however, the phenotype was unknown. ^indicates point mutations that have been identified in the literature before.



E3: Point mutations in *parC* and *parE* that were identified in PointFinder. These mutations were associated with resistance, however, the phenotype was unknown. ^indicates point mutations that have been identified in the literature before.